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INVENTORS:

Thomas J. Schall
Dale Talbot
Robert Berkovitz
Wei Zheng
Maureen Howard
Brett Premack

TITLE:

METHODS AND COMPOSITIONS FOR
INDUCING AN IMMUNE RESPONSE

ATTORNEY:

K. Shannon Mrksich
Reg. No. 36,675
Gregory M. Zinkl, Patent Agent
Reg. No. 48,492
BRINKS HOFER GILSON & LIONE
P.O. BOX 10395
CHICAGO, ILLINOIS 60610
(312) 321-4200

Patent

METHODS AND COMPOSITIONS FOR INDUCING AN IMMUNE RESPONSE

RELATED APPLICATIONS

This application claims priority to U.S. nonprovisional application 09/834,814, filed April 20, 2001, which is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

This application relates generally to enhancing immune responses. Such immune responses may be elicited by vaccine administration. Compositions and methods for inducing or enhancing an immune response to an antigen are provided. The compositions and methods are useful for, among other things, vaccine formulation for therapeutic and prophylactic vaccination (immunization) and for production of useful antibodies (*e.g.*, monoclonal antibodies for therapeutic or diagnostic use).

BACKGROUND

Immunization, or vaccination, is a widely used method to elicit an immune response to an antigen for prophylactic purposes. For example, by administering a harmless form of an antigen from a pathogen, such as an attenuated virus, the production of antibodies and stimulation of immune cells specific for the harmful form of the pathogen occurs. However, present immunization methods are not effective for all antigens. Moreover, there is a considerable lag time from immunization until the immune system provides protection for the subject. Improved methods and reagents for vaccination are desired by the medical community.

SUMMARY

The invention relates to new vaccines and immunization methods.

In one aspect, the invention is a method for eliciting an immune response to an antigen in a subject comprising administering at least one antigen-presenting cell (APC) chemotaxin and an antigen is presented. APC chemotaxins are chemotactic for immature and/or mature dendritic cells. In some aspects, APC chemotaxins are chemotractive specifically for only immature and/or mature dendritic cells.

In another aspect, the invention is a method of enhancing an immune response of a subject to an antigen comprising administering an APC chemotaxin and the antigen. The immune response may comprise an antibody-mediated response; such enhancement comprises an increase in antigen-specific antibodies by at least 2-fold.

5 The immune response may comprise a cell-mediated response.

In another aspect, the invention comprises co-administering the APC chemotaxin and antigen; alternatively, the APC chemotaxin and antigen are administered separately to elicit or enhance an immune response to the antigen.

10 In another aspect, the invention comprises administering at least two AOC chemotaxins. Such chemotaxins may be a chemokine polypeptide or variant, or a chimera of various chemokines/chemokine domains. Exemplary chemokines include hMIP1 α , hMIP1 α (70aa), mMIP-1 α , hRANTES, hMET-RANTES, mRANTES, hHCC-1, hMIPF-1, hMIPF-1 (22-137), hMIPF-1 (46-137), hMIP-1 δ , hMCP-4, mMCP-5, mMARC, mEotaxin, mMCP-1(JE), mTECK, mMIP-2, mBLC, hLeukotactin, mMIG, mMIP-1 β , hMCP-2, hMCP-3, vMIP-1, hMIP-3 α , hMIP-3 β , mC10, mMDC, hMIP-1 β , vMCK-2, mMIP-1 γ , mC10, mMIP-1 γ and vMCK-2.

In another aspect, administering APC chemotaxins includes formulating APC chemotaxins in a sustained release pharmaceutical composition.

20 In another aspect of the invention, antigens are pathogen polypeptides, such as those from *Hepatitis* or *Influenza*, or a tumor adjuvant.

In another aspect of the invention, APC chemotaxins may be administered with adjuvants, such as alum, incomplete Freund's adjuvant, a bacterial capsular polysaccharide, dextran, IL-12, GM-CSF, CD40 ligand, IFN-gamma, IL-1, IL-2, IL-3, IL-4, IL-10, IL-13, IL-18, and a cytokine. In another aspect, the invention comprises 25 multi-valent carriers that may be linked to an APC chemotaxin, antigen and/or adjuvant. Multivalent carriers may be, for example, bacterial capsular polysaccharides (such as *Pneumococci*, *Streptococci*, or *Meningococci*), a dextran and a genetically engineered vector.

In another aspect, the invention comprises administering with an APC chemotaxin a pharmaceutical carrier.

In another aspect, the invention is administering APC chemotaxins into a solid tumor or the tissue surrounding a solid tumor.

5 In another aspect, the invention is , the administering of an APC chemotaxin is accomplished by administering a polynucleotide encoding the APC chemotaxin. In another aspect, a polynucleotide encoding an antigen is administered. In other aspect, polynucleotides encoding at least one APC chemotaxin and at least one antigen are administered

10 In another aspect, the invention is a composition comprising at least one APC chemotaxin, and at least one antigen. In a another aspect, the APC chemotaxin is chemotactic for mature or immature dendritic cells; the APC chemotaxin may be specifically chemotactic for mature or immature dendritic cells; that is, the APC chemotaxin is not chemotactic for neutrophil, T cell, B cell, monocyte and/or eosinophil.

15 In another aspect, the invention is a composition comprising at least two APC chemotaxins. In another aspect, the invention is a composition comprising an APC chemotaxin that is is a chemokine polypeptide or a variant thereof. In another aspect, the invention is a composition comprising an APC chemotaxin formulated in a
20 sustained release formulation. In another aspect, the compositions of the invention further comprise at least one pharmaceutically acceptable carrier, such as an adjuvant. In other aspects, the adjuvant in the compositions of the invention is water, an oil, a saline solution, aqueous dextrose and/or glycerol solution.

25 In another aspect, the invention is an immunogenic composition comprising a cell exogenously expressing an APC chemotaxin. In a further aspect, APC chemotaxin expressing cells are allogeneic or autologous.

In another aspect, the invention is an immunogenic composition at least one APC chemotaxin and a tumor-associated antigen. Such an antigen may be from an allogeneic or autologous cell.

In another aspect, the invention is an immunogenic composition comprising at least one tumor cell, and at least one cell exogenously expressing an APC chemotaxin. A suitable tumor cell may be a primary tumor cell, such as from an autologous cell or a glioma, glioblastoma, gliosarcoma, astrocytoma, melanoma, breast cancer cell or an ovarian cancer cell. In another aspect, the invention comprises a tumor cell that is quiescent. In other aspects, the various cells of the invention comprise are allogeneic.

In another aspect, the invention is a method of formulating a composition capable of eliciting an immune response to an antigen in a subject comprising isolating a polypeptide having an activity of an APC chemotaxin, and combining the polypeptide with the antigen.

In a further aspect, the invention is a kit comprising a pharmaceutical composition comprising an APC chemotaxin and a pharmaceutically acceptable carrier, and a syringe.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows developmental pathways of dendritic cells.

Figure 2 shows the sequence of exemplary chimeric chemokines.

DETAILED DESCRIPTION

Definitions and Abbreviations

Polynucleotide

A polynucleotide refers to a deoxyribonucleotide (DNA) or ribonucleotide (RNA) in either single- or double-stranded form, and encompasses known analogs of natural nucleotides that can function in a manner similar to the naturally occurring nucleotides. The phrase "polynucleotide encoding" refers to a nucleic acid sequence that directs the expression of a specific protein or peptide. The nucleic acid sequences include both the DNA sequence that is transcribed into RNA and the RNA sequence that is translated into the protein. The nucleic acid sequence includes both the full-length nucleic acid sequence as well as non-full length sequences derived from the full-length sequence.

Subject

A is a mammal such as human patient or volunteer, an animal such as a non-human primate, rat, mouse, rodent, rabbit, and the like, an agriculturally important mammal including without limitation, a goat, pig, cow, sheep, horse, mink, and fox and the like, and pets such as dogs, cats, and the like.

Control sequences

Control sequences are DNA sequences that enable the expression of an operably-linked coding sequence in a particular host organism. Prokaryotic control sequences include promoters, operator sequences, and ribosome binding sites. Eukaryotic cells utilize promoters, polyadenylation signals and enhancers.

Operably-linked

Nucleic acid is operably-linked when it is placed into a functional relationship with another nucleic acid sequence. For example, a promoter or enhancer is operably-linked to a coding sequence if it affects the transcription of the sequence, or a ribosome-binding site is operably-linked to a coding sequence if positioned to facilitate translation. Generally, "operably-linked" means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, enhancers do not have to be contiguous. Linking is accomplished by conventional recombinant DNA methods.

Isolated nucleic acids

An isolated nucleic acid molecule is purified from the setting in which it is found in nature and is separated from at least one contaminant nucleic acid molecule. Isolated APC chemotaxin polynucleotides are distinguished from the specific APC chemotaxin polynucleotide as it exists in cells. However, an isolated APC chemotaxin polynucleotide includes APC chemotaxin polynucleotides contained in cells that ordinarily express the APC chemotaxin polypeptide where, for example, the nucleic acid molecule is in a chromosomal location different from that of natural cells.

Purified polypeptide

An "isolated" or "purified" polypeptide, protein or biologically active fragment is separated and/or recovered from a component of its natural environment.

When the molecule is a purified polypeptide, the polypeptide will be purified (1) to obtain at least 15 residues of N-terminal or internal amino acid sequence using a sequenator, or (2) to homogeneity by SDS-PAGE under non-reducing or reducing conditions using Coomassie blue or silver stain. Isolated polypeptides include those expressed heterologously in genetically engineered cells or expressed *in vitro* since at least one component of the APC chemotaxin polypeptide natural environment will not be present. Ordinarily, isolated polypeptides are prepared by at least one purification step.

Contaminant components include materials that would typically interfere with diagnostic or therapeutic uses for the polypeptide and may include enzymes, hormones and other proteinaceous or non-proteinaceous materials. Preferably, the polypeptide is purified to a sufficient degree to obtain at least 15 residues of N-terminal or internal amino acid sequence. To be substantially isolated, preparations have less than 30% by dry weight of non-APC chemotaxin contaminating material (contaminants), more preferably less than 20%, 10% and most preferably less than 5% contaminants. An isolated, recombinantly-produced APC chemotaxin polypeptide or biologically active portion is preferably substantially free of culture medium, *i.e.*, culture medium represents less than 20%, more preferably less than about 10%, and most preferably less than about 5% of the volume of the APC chemotaxin polypeptide preparation. Examples of contaminants include cell debris, culture media, and

substances used and produced during *in vitro* synthesis of APC chemotaxin polypeptide.

Of course, polypeptides and fragments of interest can be produced by any method well known in the art, such as by expression via vectors such as bacteria, viruses and eukaryotic cells. In addition, *in vitro* synthesis, such as peptide synthesis, may be also used.

Active polypeptide

An active, *e.g.*, APC chemotaxin polypeptide or, *e.g.*, APC chemotaxin polypeptide fragment, retains a biological and/or an immunological activity similar, but not necessarily identical, to an activity of a naturally-occurring (wild-type) APC chemotaxin polypeptide. Immunological activity, in the context of this immediate discussion of the polypeptide *per se*, and not an actual biological role for APC chemotaxin in eliciting or enhancing an immune response, refers to an aspect of an APC chemotaxin polypeptide in that a specific antibody against an antigenic epitope possessed by a native APC chemotaxin polypeptide binds an APC chemotaxin; biological activity refers to a function, either inhibitory or stimulatory, caused by a native APC chemotaxin polypeptide. A biological activity of APC chemotaxin polypeptide includes, for example, chemotaxis, inducing, enhancing or aiding an immune response. A particular biological assay (see Examples), with or without dose dependency, can be used to determine APC chemotaxin activity. A nucleic acid fragment encoding a biologically-active portion of APC chemotaxin can be prepared by isolating a polynucleotide sequence that encodes a polypeptide having an APC chemotaxin biological activity, expressing the encoded portion of APC chemotaxin (*e.g.*, by recombinant expression *in vitro*) and assessing the activity of the encoded portion of APC chemotaxin polypeptide.

In general, an APC chemotaxin polypeptide variant that preserves APC chemotaxin polypeptide-like function and includes any variant in which residues at a particular position in the sequence have been substituted by other amino acids, and further includes the possibility of inserting an additional residue or residues between two residues of the parent protein as well as the possibility of deleting one or more residues from the parent sequence. Any amino acid substitution, insertion, or deletion

is encompassed by the invention. In favorable circumstances, the substitution is a conservative substitution as defined above.

“APC chemotaxin polypeptide variant” means an active APC chemotaxin polypeptide having at least: (1) about 70% amino acid sequence identity with a full-length native sequence APC chemotaxin polypeptide sequence, (2) an APC chemotaxin polypeptide sequence lacking the signal peptide, (3) an extracellular domain of an APC chemotaxin polypeptide polypeptide, with or without the signal peptide, or (4) any other fragment of a full-length APC chemotaxin polypeptide sequence. For example, APC chemotaxin polypeptide variants include APC chemotaxin polypeptide polypeptides wherein one or more amino acid residues are added or deleted at the N- or C- terminus of the full-length native amino acid sequence. An APC chemotaxin polypeptide variant will have at least about 70% amino acid sequence identity, preferably at least about 71% amino acid sequence identity, more preferably at least about 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% amino acid sequence identity and most preferably at least about 99% amino acid sequence identity with a full-length native sequence APC chemotaxin polypeptide sequence. An APC chemotaxin polypeptide variant may have a sequence lacking the signal peptide, an extracellular domain of an APC chemotaxin polypeptide polypeptide, with or without the signal peptide, or any other fragment of a full-length APC chemotaxin polypeptide sequence. Ordinarily, APC chemotaxin variant polypeptides are at least about 10 amino acids in length, often at least about 20 amino acids in length, more often at least about 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, or 300 amino acids in length, or more.

“Percent (%) amino acid sequence identity” is defined as the percentage of amino acid residues that are identical with amino acid residues in an APC chemotaxin polypeptide sequence in a candidate sequence when the two sequences are aligned. To determine % amino acid identity, sequences are aligned and if necessary, gaps are introduced to achieve the maximum % sequence identity; conservative substitutions are not considered as part of the sequence identity. Optimal alignment of sequences for comparison can be conducted, *e.g.*, by the local homology algorithm of Smith & Waterman, 1981, *Adv. Appl. Math.* 2:482, by the homology alignment algorithm of

Needleman & Wunsch, 1970, *J. Mol. Biol.* 48:443, by the search for similarity method of Pearson & Lipman, 1988, *Proc. Natl. Acad. Sci. USA* 85:2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 5 575 Science Dr., Madison, WI), or by visual inspection (see generally Ausubel *et al.*, *supra*). Each of these references and algorithms is incorporated by reference herein in its entirety. When using any of the aforementioned algorithms, the default parameters for “Window” length, gap penalty, etc., are used.

When amino acid sequences are aligned, the % amino acid sequence identity 10 of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) can be calculated as:

$$\% \text{ amino acid sequence identity} = X/Y \cdot 100$$

where

X is the number of amino acid residues scored as identical matches by the sequence alignment program's or algorithm's alignment of A and B

and

Y is the total number of amino acid residues in B.

If the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A.

25 One example of algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in Altschul *et al.*, 1990, *J. Mol. Biol.* 215:403-410. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>).

30 This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of

the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Extension of the word hits in each direction are halted when the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLAST program uses as defaults a word length (W) of 11, the BLOSUM62 scoring matrix (see Henikoff & Henikoff, 1989, *Proc. Natl. Acad. Sci. USA* 89:10915) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

Substantially pure cell population

As used herein, a cell sample or cell population is “substantially pure” or “substantially purified” if at least about 75%, preferably at least about 85%, often at least about 90%, at least about 95% or more of the total cells in the sample are of a defined type (*e.g.*, in a substantially pure preparation of dendritic cells, at least about, *e.g.*, 95% of the total number of cells are dendritic cells, while in a substantially pure preparation of immature dendritic cells, at least about, *e.g.*, 95% of the total number of cells are immature dendritic cells).

Allogeneic and autologous cells

An allogeneic cell means a cell that is derived from individuals of the same species that is sufficiently unlike genetically to interact antigenically. An autologous cell means a cell that occurs normally in a tissue or body structure, referring to a cell in which the donor and recipient of the cell are the same individual.

Abbreviations

When referring to a chemokine “h” means human and “m” means murine. In some cases, Latin letters are used instead of the Greek α , β , or γ . Thus, as used herein, mMIP-1a means murine MIP-1 α .

APC chemotaxin compositions

In one aspect, the method of the invention involves administering a composition that contains an agent that is chemotactic for dendritic cells (DCs) and/or macrophages (together referred to as “antigen-presenting cells” or “APCs”). For convenience, the chemotactic agent is sometimes referred to as a “APC chemotaxin,” and the composition containing the APC chemotaxin (which may contain excipients or other components) is sometimes referred to herein as “the chemotactic composition” or “the composition.”

In some embodiments of the invention, the APC chemotaxin is specifically chemotactic for specified cell types (*e.g.*, dendritic cells) or cell developmental stages (*e.g.*, immature dendritic cells). In related embodiments the agent, while chemotactic for some cells, is *not* chemotactic for certain cell-types (*e.g.*, neutrophils) or cell developmental stages (*e.g.*, mature dendritic cells). Chemotaxis is determined using one or more of the assays described herein.

In one aspect, the methods of the invention involve administration of an antigen, in addition to a chemotactic composition. Thus, in one embodiment, the chemotactic composition and an antigen are administered at the same physical site in the subject. For example, the antigen may be combined with a chemotactic composition, and the mixture administered (*e.g.*, injected) together. Alternatively, the composition and the antigen are administered separately to the same area of the subject (*e.g.*, injected to the same site, applied topically to the same site, and the like). In some embodiments, as described in detail *infra*, the composition and antigen are administered at different times.

Without intending to be bound by a particular mechanism, it is believed that the APC chemotaxin(s) promote(s) an immune reaction to the antigen by recruiting APCs to areas of antigen contact. Antigens are taken up by APCs and partially degraded. Subsequently, a fraction of the degraded antigen is presented with MHC class I or II molecules on the surface of the APC. Such cells stimulate proliferation of either cytotoxic T cells or helper T cells, or the production of antibody by B cells.

In a related aspect of the invention, the chemotactic composition is administered without an accompanying antigen (*e.g.*, injection into a solid tumor to elicit an immune response to cancer cells, or injection in tissue surrounding a solid

tumor, *e.g.*, within 2 cm, of a solid tumor). Without intending to be bound by a particular mechanism, it is believed that the APC chemotaxin(s) promote(s) an immune reaction to the endogenous (*e.g.*, tumor) antigen by recruiting APCs to areas of antigen contact.

The invention also provides new methods and reagents useful for therapeutic and prophylactic immunization (*i.e.*, the deliberate provocation, enhancement or intensification, of an adaptive immune response). Particular advantages expected over prior immunization methods include:

- (1) an accelerated immune response in a host following administration of antigen,
- (2) a more effective response to administration of, or exposure to, very small quantities of an antigen (*e.g.*, toxin or pathogen) due to increased antigen uptake by APCs, and
- (3) more effective anti-tumor therapies (*e.g.*, due to enhanced tumor antigen uptake by host APCs).

As used herein, an “immune response” means, unless otherwise specified, an adaptive immune response to a specific antigen. In one aspect, an immune response involves the concerted action of lymphocytes, antigen presenting cells, phagocytic cells, and various soluble macromolecules in defending the body against infection or other exposure to non-self molecules. The immune response can be detected and quantified (*e.g.*, following immunization) by measuring cellular or humoral responses according to numerous assays known in the art (see, *e.g.*, Coligan *et al.*, 1991 (suppl. 1999), *Current Protocols in Immunology*, John Wiley & Sons (hereinafter, sometimes “Coligan”)). For example, to detect a cellular immune response, T cell effector effects against cells expressing the antigen are detected using standard assays, *e.g.*, target-cell killing, macrophage activation, B-cell activation or lymphokine production. Humoral responses are measured by detecting the appearance or increase in the titer of antigen-specific antibodies using routine methods such as ELISA. The progress of the antibody response can be determined by measuring class switching (*i.e.*, the switch from an early IgM response to a later IgG response. To enhance an immune response means, unless otherwise specified, to increase the vigor and/or magnitude and/or quality of the immune system being stimulated. For example, the early

appearance and/or a high titer of antigen-specific antibodies, especially when compared to controls in which the immune response was not modulated, signifies (but does not limit the invention) a vigorous (enhanced) immune response. Preferably, such magnitude is at least 2-fold, more preferably 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold and most preferably at least 10-fold when compared to control cells in which the immune response was not modulated. An enhanced immune response also includes enhancing the immune response's quality, such as can be determine, for example that is meant to be non-limiting, higher affinity antibodies to the specific antigen, a greater presence of preferred IgGs, *etc.*

Chemotactic compositions

According to the invention, a composition comprising an APC chemotaxin is administered to induce or enhance an immune response in a subject. In various embodiments, the chemotaxin attracts dendritic cells, macrophages, or both. Chemotaxins that attract dendritic cells, particularly immature dendritic cells, are especially useful. In a particularly useful embodiment, the chemotaxin has a high level of specificity for a particular cell type, developmental stage, or both. Thus, in one embodiment, the APC chemotaxin attracts immature dendritic cells, but does not attract one or more of the following other classes of cells: mature dendritic cells, neutrophils, monocytes, T cells, B cells, eosinophils, mast cells, red blood cells, and progenitor cells.

The APC chemotaxins of the invention may be any of a large variety of compounds, both naturally occurring and synthetic, organic and inorganic, and including polymers (*e.g.*, oligopeptides, polypeptides, oligonucleotides, and polynucleotides), small molecules, antibodies, sugars, fatty acids, nucleotides and nucleotide analogs, analogs of naturally occurring structures (*e.g.*, peptide mimetics, nucleic acid analogs, and the like), and numerous other compounds. In a preferred embodiment, the chemotactic compositions comprise polypeptides, such as naturally occurring chemokines, polypeptide variants, or mimetics of chemokines. In a preferred embodiment of the invention, the composition contains at least a chemokine polypeptide, such as hMCP2, hMCP3, hMIP1 β , hMIP3 α , hMIP3 β , mMIG, mMIP1 γ , mMDC, vMIP1, mC10, a variant of an aforementioned chemokine, such as a recombinant polypeptide produced by *in vitro* mutation, *in vitro* recombination or

shuffling of a polynucleotide encoding the chemokines, a polynucleotide encoding at least one of the aforementioned chemokines or variant molecules, or a synthetic (*i.e.*, chemically synthesized) polypeptide variant (*e.g.*, mimetic) of one or more of these chemokines.

5 Reagents and methods for, *inter alia*, identifying APC chemotaxins, and preparing and administering the compositions of the invention are described *infra*.

Practising the Invention

Assays for Identification of APC chemotaxins

10 In one aspect of the invention, APC chemotaxins are identified using *in vivo* or *in vitro* assays.

(a) In vitro assays

The APC chemotaxins used in the methods of the invention have certain properties, which can be detected in *in vitro* chemotaxis assays. *In vitro* chemotaxis assays are well known. Migration assays are often carried out by physically separating the cells from the chemoattractant using a porous membrane and allowing the cells to move directionally, up a diffusion gradient of the chemotaxin (see, *e.g.*, Keller, 1972, *Agents Actions* 2:161-69; Gee A.P., 1984, *Mol. Cell. Biochem.* 62:5-11; Keller *et al.*, 1974, *Antibiot. Chemother.* 19:112-25).

20 A variety of assay configurations are known and are suitable in the practice of the present invention. Most often, standard filter based assays are used because they are convenient, robust, and relatively inexpensive. These filter based assays include a classical or modified Boyden chamber and variants (see, *e.g.*, Bozarth *et al.*, 1997, *Meth. Cell Science*, 19:179-187; Frevert *et al.*, 1998, *J. Imm. Methods*, 213:41-52; Penno *et al.*, 1997, *Meth. Cell Science*, 19:189-195; O'Leary *et al.*, 1997, *Am. J. Resp. Cell and Mol. Bio.*, 16:267-274; Falk, *et al.*, 1980, *J. Imm. Methods*, 33: 239-247; Harvath, *et al.*, 1980, *J. Imm. Methods*, 37:39-45; Richards *et al.*, 1984, *Immunological Communications*, 13:49-62; Falk *et al.*, 1982, *Infection and Immunity* 36:450-454; Harvath *et al.*, 1982, *Infection and Immunity* 36:443-449; all
25 incorporated by reference). In filter-based assays, cells are placed in a compartment separated from a candidate chemotaxin by a filter through which the candidate chemotaxin can diffuse. After an incubation period, the number of cells (or
30

percentage of total cells) that have migrated onto or through the filter is determined. Migration of cells at a level above background, *i.e.*, migration in the absence of the candidate chemotaxin, *e.g.*, in the presence of only a carrier such as PBS or Chemotaxis buffer (*infra*), indicates that the candidate chemotaxin is indeed

5 chemotactic for the target cell type. Conversely, when the number of migrating cells is at or below background, the candidate chemotaxin is considered not chemotactic for the target cell type.

One suitable apparatus for carrying out *in vitro* chemotaxis assays is a 96-well ChemoTx® microplates (Neuroprobe Inc., Gaithersburg, MD). The ChemoTx®

10 instrument has a 96 well microplate of injection-molded, tissue-culture grade, transparent polystyrene. The microplate provides bottom wells for chemoattractants and other reagents. In place of top “wells,” a framed filter is used that confines each cell-suspension sample to its site on top of the filter. The 96 sites on the filter correspond to the 96 wells in the microplate, and cell suspension pipetted directly

15 onto the sites on the top side of the filter sit in hemispherical drops during incubation. After incubation the migrated cells on the filter and in the microplate are counted. (U.S. Pat. No. 5,284,753 (incorporated herein by reference in its entirety for all purposes)).

To assay for chemotactic activity using an apparatus such as the ChemoTx®

20 microplates apparatus, candidate chemotaxins are added to lower wells (*e.g.*, about 30 microliters) at one or more concentrations (*e.g.*, about 1 nM, about 10 nM, about 100 nM, about 10 ng/ml, about 100 ng/ml and/or about 1 µg/ml), and cells are placed on porous polycarbonate filters positioned above the chemotaxin solution in each well. The filters have a pore size of about 3 µm, or about 5 µm, so that only cells exposed to

25 an agent chemotactic for the cell will migrate into and/or through the filter. In the microplate configuration, about 20 µl of cells (at about 1×10^6 to about 1×10^7 cells per ml, *e.g.*, 5×10^6 cells per ml) are usually used. The cells are incubated for a period of time (*e.g.*, 0.5 to 6 hours, usually about 1.5 h, at from about 22°C to about 39°C, usually 37°C).

30 When *in vitro* assays are conducted using a purified cell population (*e.g.*, dendritic cells, neutrophils, immature dendritic cells, *etc.*) the assay conditions are usually tailored to the cell type being studied. For example, cells that are relatively

plastic are able to crawl through certain filters, where as others become “stuck” in the filter. Thus, monocytes exposed to a monocyte chemotactic agent migrate through a 5 μm filter into the lower chamber. In contrast, immature or mature dendritic cells exposed to a chemotactic agent migrate into, and are retained in, a 3 μm or 5 μm filter.

5 Therefore, the choice of migration assay format and the method of quantification can vary depending on the type of cells studied. For example, a 5 μm pore size is usually used for a monocyte migration assay; the assay is typically carried out for 90 minutes, and the migrating cells (if any) are detected in the well. For mature dendritic cells, a 3 μm pore size filter and 90 minutes incubation are used, and the cells are detected in
10 the filter. These and other exemplary assay conditions are shown in Table 1.

However, it will be appreciated that a number of different assay formats and conditions can be used to determine that an agent does (or does not) have chemotactic activity (*e.g.*, for a particular cell). Table 1 also provides exemplary positive and negative controls for use in assays. Positive controls are agents that, when used at
15 100 nM concentration, have chemotactic activity. Negative controls are agents that, when used at 100 nM concentration, do not have chemotactic activity.

Table 1 Exemplary assay parameters and positive and negative controls for *in vitro* chemotaxis assays

cell type	filter (pore size)	time (minutes)	location	+ con.	- con.
monocyte	5 μ m	90	well	RANTES	MIP1b
immDC	5 μ m	90	filter	RANTES	SLC
matDC	3 μ m	90	filter	SLC	RANTES
neutro	3 μ m	60	well	IL8	SLC
eosino	3 μ m	60	well	Eotaxin	IL8
T cell	3 μ m	180	well	MDC	Eotaxin
B cell	3 μ m	180	well	SLC	Eotaxin
Macrophage	5 μ m	90	filter (+well)	RANTES	SLC
Key: +con. (positive control), -con. (negative control) are used at 100 nM concentrations.					

To determine the number of cells that have migrated in the presence of an agent, a variety of methods can be applied. To assay cells (*e.g.*, dendritic cells) that are caught in a filter, the filters are scraped to remove nonadherent cells (*i.e.*, cells that have simply settled onto the top side of the filter), and the cells that have moved into the filter or adhered to its lower surface are quantitated. For cells that migrate through the filter (*e.g.*, monocytes), the filter can be discarded, and the number of cells that have migrated through the filter into the “lower” (chemotaxin-containing chambers and control chambers) is determined. Suitable cell quantitation methods are well known and include direct (microscopic) counting of migrated cells, histological, cytochemical, immunofluorescence (using antibodies to cell-specific or stage-specific markers), *in situ* staining methods, use of radiolabeled cells, and assays for RNA or DNA content (*e.g.*, using reagents such as CyQuant, Molecular Probes, Eugene OR, *e.g.*, on a lysed cell extract), protein content (*e.g.*, using stains such as Hema3), enzyme activity (*e.g.*, β -glucuronidase), and the like. Often it is convenient to select stains that can be detected using densitometric or fluorescence plate readers.

In some embodiments, several concentrations of the candidate chemotaxin are used to detect an active concentration, *e.g.*, at least two, typically at least three different concentrations, over a range of at least 10-fold, typically at least 100-fold, frequently at least 1,000-fold, and often at least 10,000-fold differences.

The chemotaxis assays can be carried out using a heterogeneous mixture of cells (*e.g.*, peripheral blood mononuclear cells (PBMCs)) or a purified subpopulation (*e.g.*, immature DCs, mature DCs, neutrophils, monocytes and the like) or tissue culture cells derived from a certain cell type and with characteristics of those cells (*e.g.*, THP1 (an acute monocytic leukemia cell line), CEM acute lymphoblastic leukemia, T lymphoblast cell line). When a heterogeneous mixture of cells is used, the cell type or developmental stage of migrating cells is usually determined (*e.g.*, based on morphology, histology, or staining specific markers). When homogeneous cell preparations (*e.g.*, substantially purified neutrophils), are used, any cells that migrate to the chemotaxin can be presumed to be of that type.

In various embodiments, a candidate chemotaxin is considered chemotactic for a particular cell type if the candidate chemotaxin, at a concentration about between about 1 pM and about 1 μ M, *e.g.*, between about 1 nM and 500 nM, *e.g.*, 1 nM, about 10 nM, about 100 nM, or between about 1 pg/ml and about 10 μ g/ml, *e.g.*, between about 1 ng/ml and 1 μ g/ml, *e.g.*, about 10 ng/ml, about 100 ng/ml or about 1 μ g/ml, attracts the cell at least 2-fold, preferably at least 4-fold, and often at least 8-fold more than “chemotaxis buffer” (a negative control) in an *in vitro* assay. Chemotaxis buffer is 0.1% BSA (Sigma; St. Louis, MO) in Hank’s Balanced Salt Solution (HBSS; Life Technologies; Gaithersburg, MD; CaCl₂ (0.14g/l), KCl (0.4g/l), KH₂PO₄ (0.06g/l), MgCl₂·6H₂O (0.1g/l), MgSO₄·7H₂O (0.1g/l), NaCl (8g/l), NaHCO₃ (0.3g/l), Na₂HPO₄ (0.048g/l), D-glucose (1g/l)), with 1.4 mM Ca⁺⁺ and 1 mM Mg⁺⁺. An alternative negative control is PBS. A candidate chemotaxin is considered not to be chemotactic for a cell type if the candidate chemotaxin at a concentration of 1 nM, 10 nM, 100 nM, or 10 ng/ml, 100 ng/ml or 1 μ g/ml, does not attract more cells (*e.g.*, at least as many, sometimes at least 1.5-fold as many) as the negative control in an *in vitro* assay. In various embodiments, a candidate chemotaxin is considered chemotactic for a particular cell type if the candidate chemotaxin, when injected *in vivo*, *e.g.*, intradermally into a mouse or monkey, at 2 μ g, alternatively 10 μ g, often 20 μ g,

attracts the cell at least 2-fold, preferably at least 5-fold, and often at least 10-fold more than a negative control, such as PBS (KCl (0.2g/l), KH₂PO₄ (0.2g/l), NaCl (8.0g/l), Na₂HPO₄ (2.16g/l)). In various embodiments, a candidate chemotaxin is considered not chemotactic for a cell type if the candidate chemotaxin, when injected at 2 µg, alternatively 10 µg, often 20 µg, does not attract more cells of the type (*e.g.*, at least as many, sometimes at least 1.5-fold as many) than the negative control in an *in vivo* assay.

In some embodiments, an agent is determined to be chemotactic because when having greater chemotactic activity than a known chemotaxin (*e.g.*, known in the art or determined by the assays described herein).

Non-filter based chemotaxis assays may also be used, *e.g.*, a cell migration assay can be carried out using a monolayer of cells grown on the filter as a barrier. In other examples, cellular motility may also be assessed by monitoring the movement of a single cell under a video microscope. In these types of experiments, the chemoattractant is applied through a capillary, and the physical distance of cell's lateral movement toward the source of the chemoattractant is recorded.

(b) *In vivo* assays

In one embodiment, the chemotactic properties of an agent can be determined in animals, *e.g.*, mammals such as non-human primates and mice, as described in Examples 2–4, *infra*. In one *in vivo* assay, the candidate chemotactic agent (*e.g.*, 2-20 µg in PBS) is administered by intradermal injection into an animal. After a period of time (*e.g.*, 24 hours, 72 hours, 96 hours), the animal is euthanized or a biopsy is taken. The area around the injection site is excised and subjected to routine histology or immunohistology techniques to determine the presence or absence of cell infiltration and, if an infiltrate is present, to characterize and quantitate the infiltrating cells (*e.g.*, mononuclear cells, neutrophils, dendritic cells, etc.). For general histological methods, see, *e.g.*, *The Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology*", by Lee G. Luna, McGraw-Hill, 3rd edition, 1968 (hereinafter "Luna"). In one embodiment, cells are characterized by preparing frozen sections and staining with cell type specific antibodies, or cell type-specific combinations of antibodies, using well known methods (see, Harlow *et al.*, 1988, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory).

Characterization of Cell Types and Preparation of Purified Cells

Enriched or substantially purified cell populations can be used in *in vitro* chemotaxis assays. These cell populations can be prepared by a variety of methods known in the art depending on the specific cell-type desired. Typically, substantially purified cell populations are prepared by culture under specific conditions, by physical characteristics such as behavior in a density gradient, by sorting according to characteristic markers (*e.g.*, by fluorescence activated cell sorting (FACS) using antibodies (preferably monoclonal antibodies) to cell-surface proteins, immunoprecipitation), or other methods.

Cells can be identified by histology (see, *e.g.*, Luna, *supra*), by immunological staining and similar methods (see, *e.g.*, Harlow, *supra*; Coligan *et al.*, *supra*). Table 2A and 2B list exemplary markers useful for characterizing or purifying (*e.g.*, FACS) certain immune system cells. Many other markers are known in the art, both for the cells listed and for other immune system cells such as B cells, T cells, neutrophils, eosinophils, and others (see, *e.g.*, Janeway-Travers, 1994, *ImmunoBiology* Garland Pub., N.Y.; Paul, *Fundamental Immunology* 3rd Ed, Raven Press N.Y. 1993). Table 2A shows certain classical cell surface markers; Table 2B shows certain chemokine receptors expressed by specific cell types.

Table 2A Selected Cell Surface Markers

Marker	CD3	CD14	CD20	CD25	CD68	CD80	CD83	CD86	HLA-DR	CD1a
Monocyte-derived Immature DCs	-	-	-	-	+	-	-	-	low	high
Mature DCs	-	-	-	+		+	+	high	high	+
Macrophages		high			+					-
Monocytes	-	+	-	-		-	-	low	low	-

Key: Table 2A shows the levels of markers as determined by staining with an antibody specific for the marker. “-“ indicates staining equivalent to an isotype control antibody (an isotype control antibody is the same isotype as the staining antibody, but does not recognize the assayed epitope); “+” indicates at least 10 fold higher levels of staining as isotype control; “low” indicates staining 2-5 fold higher than isotype control; “high” indicates staining 10 to 1000 fold higher than isotype control.

Table 2B Chemokine Receptor Expression Profile of Specific Cell Types

	CC receptors						CXC receptors					XCR1
	R1	R2	R3	R5	R6	R7	R1	R2	R3	R4	R5	
Immature DCs	+	-	-	+	-	-	-	-	-	+	-	-
Mature DCs	-	-	-	-	-	+	-	-	-	+	-	-
Monocytes	+	+	-	-	-	-	-	-	-	+	-	-

- 5 Methods for preparing substantially purified cell compositions for use in *in vitro* chemotaxis assays are briefly described *infra* and in the Examples. However, the invention does not require that any particular purification method be used, so long as the desired cells are obtained; many variations and alternative methods are known to those of skill in the art. Further, many other purification and detection methods,
- 10 including methods suitable for cells not specifically listed herein, are known in the art or can be easily developed. Further, cloned cell lines derived from immune system

tissues can be used in the chemotaxis assays described herein, if desired. General immunological, purification and cell culture methods are described in Coligan *et al.*, and (1991) *Current Protocols in Immunology*, John Wiley & Sons, including supplements through 1999, incorporated herein by reference in its entirety for all purposes. Unless otherwise specified, cells in culture are incubated at 37°C in 5% CO₂.

(a) *Monocytes*

Suitable methods for monocyte purification are found in Bender *et al.*, 1996, *J. Immunol. Methods* 196:121-35 (also see U.S. Pat. No. 5,994,126). Briefly, monocytes are isolated from PBMC by depleting T cells using immobilized antibodies against a pan T cell surface marker CD2. Conveniently, a commercially available source of CD2 antibodies attached to magnetic beads (Dynal; Lake Success, NY) is used. PBMC isolated from a buffy coat (typically 35 mls containing 400 x 10⁶ PMBC) by conventional Ficoll gradient centrifugation methods are resuspended in MACS buffer (DPBS (HyClone; Logan, UT) with 1% BSA (Sigma)) at 20 x 10⁶ cells per ml. DPBS is Dulbecco's Phosphate Buffered Saline (CaCl₂ (0.1g/l), KCl (0.2g/l), KH₂PO₄ (0.2g/l), MgCl₂-6H₂O (0.1g/l), NaCl (8.0g/l), Na₂HPO₄ (2.16g/l)). An appropriate amount of immobilized CD2 + magnetic beads (typically 10 µl per 10⁶ cells) are added to the cells. The mixture is incubated for 15 minutes at 4°C with gentle rotation. The magnetically tagged T cells are removed from the unlabeled cells on a magnetic cell sorter (Dynal) according to the manufacturer's protocols. The unlabeled cells contain primarily monocytes and B cells.

B cells in the above preparation are removed by taking advantage of differential adhesion properties. Briefly, PBMC depleted of T cells are allowed to adhere to the plastic of a T-175 tissue culture flask (100 x 10⁶ cells/ flask; Costar; Acton, MA) for 3 hours at 37°C. Non-adherent cells (comprising largely B cells) are aspirated. To completely remove non-adherent cells, the flasks are rinsed 3 more times with DPBS. The resulting cells are largely enriched (*i.e.*, > 90%) for monocytes.

Monocytes can also be isolated by positive selection of CD14 antigen. Briefly, PBMC isolated from peripheral blood, such as a buffy coat, by standard

Ficoll gradient centrifugation methods are resuspended in MACS buffer at 1×10^6 cells/ml. Immobilized antibodies against the CD14 surface antigen, such as CD14+ magnetic microbeads (Miltenyi) are added (1 μ l of beads per 1×10^6 cells) and the mixture is incubated at 4°C for 15 minutes. Monocytes are separated from the other cell populations by passing the mixture through a positive selection column on a magnetic cell sorter (Miltenyi Biotech; Auburn, CA) according to manufacturers protocol. Monocytes that are retained on the column are eluted with MACS buffer after the column is removed from the MACS apparatus. Cells are then pelleted by centrifugation and resuspended in RPMI plus 10% FCS media at 10^6 cells per ml. Monocytes isolated by this method are cultured essentially the same way as those isolated by the CD2+ depletion method.

(b) *Preparing Purified Dendritic Cell Populations*

Suitable methods for purification of dendritic cells, including separate mature and immature populations, are known in the art. Substantially purified dendritic cells (including subpopulations of mature or immature cells) can be prepared by selective *in vitro* culture conditions.

Dendritic cells are widely distributed in all tissues that have contact with potential pathogens (e.g., skin, gastrointestinal and respiratory tracts, and T cell-rich areas of the secondary lymphoid tissues). In the skin and upper respiratory tract they form a lattice of highly arborised cells (called Langerhans cells in the skin). After capturing antigen, dendritic cells in the peripheral tissues such as the skin and gut, traffic via the draining lymphatics to the T cell areas of lymph nodes where they present the internalized antigen. Immature dendritic cells function to take up and process antigens. During subsequent migration to the draining lymph node, the DC matures. The mature dendritic cells functions as the key APC to initiate immune responses by inducing the proliferation of pathogen specific cytotoxic and helper T cells.

Substantially pure populations of dendritic cells can be produced by *in vitro* culture, *infra*). In addition, there are marked changes in expression of chemokine receptors during dendritic cell maturation which can be used to identify cell stage (Campbell *et al.*, 1998, *J Cell Biol* 141:1053; Chan *et al.*, 1999, *Blood* 93:3610; Dieu

et al., 1998, *J Exp Med* 188:373; Kellermann *et al.*, 1999, *J Immunol* 162:3859). For example, immature dendritic cells express predominately CCR1, CCR5, and CXCR4. Upon maturation, these receptors, with the exception of CXCR4, are down regulated (*See also*, Table 2B).

In culture, immature forms of dendritic cells undergo maturation thought to be analogous to the events during migration of dendritic cells from the point of antigen contact until to the secondary lymphoid tissues. Human or macaque dendritic cells of various developmental stages can be generated in culture, from CD14⁺ blood progenitors using specific cytokines. A separate lineage of dendritic cells can be differentiated from CD34⁺ precursor cells from cord blood or bone marrow. In one embodiment of the invention, subpopulations of dendritic cells are generated for *in vitro* assays for identification of chemotactic compositions (*i.e.* to assess chemotaxin potency and selectivity against defined DC sub-types). Exemplary subpopulations of dendritic cells are: (1) immature peripheral blood monocyte derived cells; (2) mature peripheral blood monocyte derived cells, and (3) cells derived from CD34⁺ precursors. Subpopulations are isolated or produced by a variety of methods known in the art. For example, immature and mature dendritic cells from PBMCs are produced according to Bender *et al. supra*.

(i) *Immature Dendritic Cells*

Briefly, PBMCs are depleted of T cells using immobilized antibodies against the cell surface marker CD2 (present on all T cells). Commercially available CD2⁺ dynabeads (Dyna) can be used according to manufacturer's protocol. The T-cell depleted mixture is separated into adherent versus non-adherent fractions by incubating the cells on tissue culture grade plastic for 3 hours at 37°C. Non-adherent cells are gently removed, and adherent cells (generally CD14⁺ monocytes) are placed in culture media (*e.g.*, RPMI + 10% FCS) supplemented with 1000 U/mL each of GM-CSF and IL-4 (R&D Systems, Minneapolis, MN) ("Day 1"). Between days 3-7 the cells begin to display a veiled morphology, and cytokines are replenished on days 2, 4, and 6, at which time the cells can be harvested as immature dendritic cells. In one embodiment, cells of this *in vitro* stage are isolated and used in the assay. Approximately 10×10^6 dendritic cells are typically obtained from 400×10^6 PBMCs.

Day 7 immature dendritic cells exhibit typical dendritic cell morphology, with elongated cell body and many processes. The size of the cells increase significantly compared to the precursor monocytes. Immature dendritic cells can be characterized phenotypically by monitoring their expression of cell surface markers.

(ii) *Mature Dendritic Cells*

Immature dendritic cells (generated from peripheral blood monocytes or from bone marrow derived CD34+ precursors) can be further activated and differentiated to become mature dendritic cells. Two methods are primarily used: MCM (macrophage conditioned medium) and double-stranded RNA-poly (I:C) stimulation (Cella et al, 1999, *J Exp Med.* 189:821-9; Verdijk *et al.*, 1999, *J Immunol.* 1999 1:57-61).

In the MCM method, day 6 immature dendritic cells are harvested by centrifugation and resuspended in at 10^6 cells/ml in maturation medium (*e.g.*, MCM diluted (up to 1:1 with RPMI containing 10% FCS). GM-CSF (1000 U/ml) and IL-4 (1000 U/ml) are added. Cells are cultured for three more days, without further addition of GM-CSF (1000 U/ml) and IL-4. Day 9 cells are used as mature dendritic cells.

In the poly (I:C) method, day 6 immature dendritic cells are harvested and resuspended in the standard culture medium (RPMI plus 10% FCS) supplemented with 20 μ g/ml of poly (I:C) (Sigma), 1000 U/ml of GM-CSF and IL-4. Cells are cultured for another three days without additional cytokines. Day 9 cells are used as mature dendritic cells.

Mature dendritic cells generated by these two different methods exhibit phenotypic and functional properties distinct from those of immature dendritic cells or the precursor monocytes (See Table 2A). Mature dendritic cells from each preparation are thoroughly characterized by FACS to ensure that the desirable cell types are obtained.

Notably, generated mature dendritic cells express significantly higher level of MHC class II on the cell surface than immature cells. Expression of CD80, CD83 and CD86 are also up-regulated. Chemokine receptor expression also changes dramatically during the maturation process. For instance, CCR1, CCR5 are down-regulated sharply in mature cells, while CCR7 is up-regulated and appears on the cell surface within a few hours after addition of MCM. Functionally, mature dendritic

cells are no longer capable of efficiently taking up antigen, but gain the ability to stimulate the proliferation of naïve T cells and B cells. Mature dendritic cells also change their migratory behaviors; they no longer respond to ligands for CCR1, CCR2 and CCR5, such as MIP-1 α , RANTES and MIP-1 β . Instead, they respond to CCR7 ligands SLC and ELC.

MCM Media

MCM is prepared by as described by Romani *et al.*, 1996, *J. Immunol. Methods* 196:137) with minor modifications. Briefly, petri dishes (100 mm, Falcon) are coated with 5 ml of human Ig (10 mg/mL) for 30 min at 37°C and washed with PBS 2-3 times immediately before use. 50 x10⁶ PBMC in 8 ml are layered onto human Ig-coated plates for 1-2 hours. Non-adherent cells are washed away and discarded. The adherent cells are incubated in fresh complete medium (RPMI + 10% normal human serum) at 37°C, and the resulting media (MCM) is collected after 24 hours. The TNF- α concentration in the MCM is determined by the standard ELISA method (*e.g.*, using a TNF- α ELISA kit (R&D Systems, Minneapolis, MN)). The final TNF- α level in MCM is adjusted to 50 U/ml by mixing an appropriate amount of MCM with RPMI/10% fetal calf serum.

(c) *Neutrophils*

Suitable methods for neutrophil purification are known in the art. According to one suitable method, whole fresh blood (WB) is diluted 1:1 with 3% dextran in a 50 ml centrifuge tube and allowed to sediment for 30 - 45 minutes at room temperature. Twenty-five ml of WB plus 25 ml dextran results in approximately 35 ml of supernatant after 30 minutes sedimentation. The supernatant is layered over 12-15 ml Ficoll and centrifuged at 400 x g for 30-40 minutes at 18-20°C. The plasma/platelet layer containing mononuclear cells and Ficoll-Paque are removed by aspiration. Neutrophils are found in the white layer above the erythrocyte (RBC) layer. (In some preparations, the neutrophil and erythrocyte layers are mixed. In these cases, RBCs are removed by hypotonic lysis: 12.5 ml of cold 0.2% NaCl is added to the neutrophils/RBC pellet while vortexing. 12.5 ml of cold 1.6% NaCl is immediately added while still vortexing. The cells are centrifuged at 60- 100 x g for

10 m and recovered. If necessary the lysis step is repeated). The resulting neutrophils are >95% pure (with the eosinophils as the primary remaining cells).

(d) *Macrophages*

5 Suitable methods for purification of macrophages are known in the art. One suitable method is described in Paluka *et al.* (1998) *J Imm.* 9:4587-95, which is incorporated by reference herein in its entirety for all purposes.

(e) *T Cells*

10 Suitable methods for purification of T cells are known in the art. T lymphocytes are routinely prepared by removal of monocytes from the PBMC prepared by standard Ficoll gradient centrifugation methods (Coligan, *supra*). Monocytes are removed by allowing PMBC to adhere to tissue culture flasks. The non-adherent cells (lymphocytes) are cultured in RPMI/10% FCS in the presence of PHA (5 µg/ml) and human recombinant IL-2 (20 ng/ml) for two weeks, and the cells are harvested.

(f) *B Cells*

20 Suitable methods for purification of B cells are known in the art. Highly purified B cell populations are isolated by negative selection with sequential depletion of monocytes/natural killer (NK) cells and T cells (as described in *Current Protocols in Immunology*, *supra*). Depletion of monocytes and NK cells are carried out by using L-leucine methyl ester (L-LME). Briefly, PBMC isolated from peripheral blood (*e.g.*, from a buffy coat) by standard Ficol gradient methods are resuspended at 25 3×10^6 cells/ml in PBS. Freshly prepared L-LME (0.05 M solution in RPMI, no serum) is added to cells at 1:10 dilution (5 mM final). The mixture is incubated for 35 minutes at room temperature, the cells are pelleted by centrifugation, followed by washing 3x with PBS. T cells are further depleted by rosetting with neurominidase-treated sheep red blood cells (NSRBC): cells are adjusted to 10^7 cells/ml in 30 RPMI/10% FCS. Five ml of cells are transferred into a 50 ml centrifuge tube. Then, 2.5 ml of fetal calf serum and 2.5 ml of NSRBC are added to the tube. The mixture is incubated for 10 minutes at 37°C. The cells are then centrifuged at 150 x g at room

temperature for 10 minutes to pellet the cells and promote rosette formation. The mixture is incubated at 4°C for 2 hours. The pellet is gently resuspended, and the cell suspension is underlaid with Ficol (10ml). The tube is centrifuged at 400xg for 25 minutes. B cells in the interface are removed, pelleted and washed three times with HBSS. After repeating the rosetting step, B cells are resuspended in RPMI/10% FCS for migration assays.

(g) *Eosinophils*

Suitable methods for purification of eosinophils are known in the art. One method for preparation of substantially purified eosinophils is by further isolation from the preparation described in the neutrophil isolation protocol, *supra*. Separation of eosinophils from neutrophils is achieved by a negative selection method, where immobilized antibodies against the CD16 surface antigen are used to deplete the CD16 positive neutrophils. Briefly, the neutrophil preparation is resuspended in MACS buffer (1% BSA in DPBS) at a density of 10^6 cells/ μ l. An equal volume of CD16+ microbeads (CD16 immobilized on magnetic spheres; Miltenyi Biotech; Auburn, CA) is mixed with the cells. The mixture is incubated at 4°C with gentle shaking for 30 minutes. Cells are then passed through a negative selection column to remove the magnetically labeled neutrophils (CS column, Miltenyi Biotech; Auburn, CA) LC, Miltenyi) and the column is washed with one column volume of MACS buffer. The flow-through and wash fractions contain eosinophils and are combined. Eosinophils isolated by this method are more than 95% pure (as determined by FACS, *e.g.*, for the presence of the CD16+ cells).

Chemokines as Chemotactic Compositions

The APC chemotaxins of the invention may be any of a number of types of compounds. Often, the chemotaxin is a polypeptide, such as a chemokine, or a protein mimetic. Thus, in an embodiment, the chemotactic composition comprises one or more chemokines, chemokine analogues, or chemokine-encoding polynucleotides.

Chemokines are protein hormones that, among other activities, direct trafficking of white blood cell populations, *e.g.*, in the primary lymphoid organs,

blood, tissues, secondary lymphoid organs, lymph, and (in some instances), back into circulation. Over 50 distinct chemokines have been identified to date in humans, and numerous chemokines from other mammals and chemokines encoded by mammalian viruses are known.

5 Structurally, known chemokines are divided into four classes: CC, CXC, C, and CX3C, based on the number and spacing of the amino-terminal cysteine residues in a conserved structural motif. Chemokines exert promigratory effects by binding to an array of cell surface receptors on the surface of target leukocytes. Known
10 receptors are of the seven transmembrane spanning, G protein coupled receptor (7TM GPCR) class. Of the almost 20 human chemokines receptors characterized (*i.e.*, those receptors for which a ligand has been identified and binding and/or signaling events are well characterized), nine are CC chemokine receptors (CCR), six are CXC chemokine receptors (CXCR), one is a CX3C chemokine receptor (CX3CR), and one is a C chemokine receptor (provisionally 'XCR'). In addition, one promiscuous
15 chemokine receptor of broad binding specificity, originally known as the Duffy blood group antigen (Duffy Ag, sometimes denoted 'DARC') is known.

Chemokine proteins can be obtained from suppliers, *e.g.*, R&D Systems (Minneapolis, MN), or may be prepared using routine techniques based on published sequences (*e.g.*, as described in Sambrook *et al.*, 1989, *Molecular Cloning: A*
20 *Laboratory Manual*, (2nd ed.) Vols. 1-3, Cold Spring Harbor Laboratory and Ausubel *et al.*, 1999, *Current Protocols in Molecular Biology*, Greene Publishing and Wiley-Interscience, New York). For recent reviews on chemokines, see Ward *et al.*, 1998, *Immunity* 9:1-11 and Baggiolini *et al.*, 1998, *Nature* 392:565-568, and the references cited therein. Also see the CFB (*Cytokine Facts Book*, 1997, Academic Press Ltd.)
25 and the GenBank databases. Additional references are provided *infra*.

The chemokines used in, or to prepare, the compositions of the present invention may be natural (*i.e.*, have the sequence of a naturally occurring chemokine), may be the product of *in vitro* recombination of polynucleotides encoding naturally occurring chemokines, or may be synthetic (*i.e.*, chemically synthesized) or
30 recombinant variants of naturally occurring chemokine sequences. Chemokines comprise sequences from human, primate, rodent, viral, and other species. In some embodiments, xenogeneic sequences are used in the vaccination methods of the

invention (*e.g.*, if the most potent chemokine is from a species other than the species of the subject being immunized) because any immunogenic effects will likely enhance the adjuvant activity.

5 Exemplary Chemokine Compositions

In embodiments of the invention, the compositions contain at least one chemokine polypeptide that is an APC chemotaxin. In an embodiment, the chemokine is chemotactic for human immature (antigen uptake competent) monocyte derived dendritic cells.

10 To determine the chemotactic profile of a comprehensive set of known chemokines, *in vitro* chemotaxis assays were performed. A number of known chemokines are chemotactic for immature but not mature dendritic cells, including: hMIP1 α , hMIP1 α (70aa), mMIP-1 α , hRANTES, hMET-RANTES, mRANTES, hHCC-1, hMPIF-1, hMPIF-1 (22-137), hMPIF-1 (46-137), hMIP-1 δ , hMCP-4, 15 mMCP-5, mMARC, mEotaxin, mMCP-1(JE), mTECK, mMIP-2, mBLC, hLeukotactin, mMIG, and mMIP-1 β . Other particularly useful chemokines of the invention include hMCP-2, hMCP-3, vMIP-1, hMIP-3 α , hMIP-3 β , and vMCK-2 (see Examples, *infra*). Several chemokines are chemotactic for immature dendritic cells but not chemotactic for neutrophils and other cell types tested in the *in vitro* assays 20 (mC10, mMDC, hMIP-1 β , mMIP-1 γ ; Table 3). These chemokines and their variants and derivatives are especially useful in the compositions and methods of the invention.

Table 3 Immature Dendritic Cell-Specific Chemotaxins

	Immature DCs	Mature DCs	Monocytes	Neutrophils	Eosinophils	CEM (T-Cell line) ¹
mC10	+	-	-	-	-	-
mMDC	+	-	-	-	-	-
hMIP-1 β	+	-	-	-	-	-
mMIP-1 γ	+	-	-	-	-	-

¹ATTC No. CCL-119.*Homologs and Variants of Naturally Occurring Chemokines*

In one embodiment, an APC chemotaxin molecule has the sequence of a naturally occurring chemotaxin molecule, or has an amino acid sequence with substantial amino acid sequence identity to, the sequence of a naturally occurring chemotaxin molecule. For example, chemokine polypeptides, such as the chemokines listed *supra*, can be modified in ways that do not change the chemotactic properties of the naturally occurring polypeptide, for example, by conservative amino acid substitutions, truncations (especially at the termini), small internal deletions, insertions, and the like. Such modifications can be made using routine genetic engineering techniques, *e.g.*, site-directed mutagenesis, and the resulting mutants assessed for chemotactic properties (*e.g.*, using the assays disclosed herein).

In addition, recombinant and synthetic techniques can be used to modify naturally occurring chemokine molecules or sequences (including, but not limited to those listed *supra* and in Table 3) to modify the chemotactic properties of the polypeptide compared to the parent polypeptide(s).

Engineered chemokines

In one aspect of the invention, synthetic, genetically engineered or recombinant chemotaxins are prepared and assessed for the ability to mobilize APCs (*e.g.*, dendritic cells). In one embodiment, APC chemotaxins are generated from a combination of natural chemokines using synthetic or recombinant DNA technology. For example, different chemokines (*e.g.*, human, viral, murine, and the like) can be recombined (*e.g.*, genetically) to form chimeras or “hybrikinines” that are tested for the desired activity (*e.g.*, the ability attract immature dendritic cells but not neutrophils, enhanced chemoattractant activity at lower concentrations). In a related embodiment, the chimeras are chemically synthesized based on the sequence of parent (*e.g.*, naturally occurring) chemokines.

In one embodiment, the sequences of chemokine polypeptides of interest are divided into four ‘domains’ as dictated by the spacing of the cysteine residues (see Figure 2 and Examples). Alternatively, the sequences are divided into multiple “domains” (*e.g.*, 2, 3, 4, or more) of any length (but typically at least 5, more often at least 10 residues), for the purpose of constructing hybrids. The sequences are conceptually recombined to form chimeric sequences in which one region has a sequence of a first chemokine polypeptide and a second region has the sequence of a second chemokine polypeptide, as illustrated in Figure 2. Chimeric polypeptides (hybrikinines) having the chimeric sequence are then produced by routine synthetic means (or alternatively, by using recombinant DNA techniques, as described *infra*). Polypeptide synthetic methods are well known in the art and are described in, *e.g.*, U. S. Pat. No. 4,108,846; see also, Caruthers *et al.*, 1980, *Nucleic Acids Res. Symp. Ser.*, 215-223; Horn *et al.*, 1980, *Nucleic Acids Res. Symp. Ser.*, 225-232; Roberge, *et al.*, 1995, *Science* 269:202). If desired, short polypeptides may be fused by condensation of the amino terminus of one molecule with the carboxyl terminus of the other molecule to form a peptide bond to produce a longer polypeptide. The newly synthesized peptide can be substantially purified, for example, by preparative high performance liquid chromatography (*e.g.*, Creighton, 1983, *Proteins, Structures, and Molecular Principles*, W.H. Freeman and Co, New York NY).

In an alternate embodiment, the polynucleotides encoding parent chemokines of interest are manipulated to produce variant or chimeric chemokines, such as those

described *supra* (*e.g.*, the polynucleotide is divided into multiple 'domains' as dictated by the spacing of the cysteine residues of the encoded polypeptide and hybrid gene constructs) or chemically synthesized polynucleotides are prepared. The hybrid genes are subcloned into appropriate expression vectors and introduced (*e.g.*,
 5 transfected) into host cells (*e.g.*, bacterial or eukaryotic cells) and the cells are cultured under conditions in which the recombinant protein is expressed. Supernatants of transfected cells are assayed for the desired chemoattractant properties using the assays described *supra*. Techniques for nucleic acid manipulation and expression are described generally, *e.g.*, in Sambrook, *et al.*, 1989, *Molecular Cloning: A Laboratory*
 10 *Manual* (2d ed.), vols. 1-3, Cold Spring Harbor Laboratory; and Ausubel, *et al.* (eds.) (supplemented to 1999) *Current Protocols in Molecular Biology*, Greene and Wiley, NY.

In an embodiment, a chimeric molecule comprises at least 10 contiguous residues from each of at least two different naturally occurring chemokines. In an
 5 embodiment, at least one of the naturally occurring chemokines, often two, and sometimes at least three or more of the chemokines, are selected from: hMIP1 α , hMIP1 α (70aa), mMIP-1 α , hRANTES, hMET-RANTES, mRANTES, hHCC-1, hMPIF-1, hMPIF-1 (22-137), hMPIF-1 (46-137), hMIP-1 δ , hMCP-4, mMCP-5, mMARC, mEotaxin, mMCP-1(JE), mTECK, mMIP-2, mBLC, hLeukotactin, mMIG,
 20 and mMIP-1 β , hMCP-2, hMCP-3, vMIP-1, hMIP-3 α , hMIP-3 β , vMCK-2, and especially, mC10, mMDC, hMIP-1 β , and mMIP-1 γ . In an embodiment, the at least two different naturally occurring chemokines are from different species.

Derivatives of naturally occurring chemokines with enhanced dendritic cell attractant properties and decreased recruitment of non-dendritic immune cells, are
 25 constructed using different hybrids of human and viral chemokines. For example, an APC chemotaxin (*e.g.* a chemokine) with potent dendritic cell chemotactic activity but undesired neutrophil chemotactic activity can be recombined with a polypeptide with weaker dendritic cell activity and no neutrophil chemotactic activity to produce a polypeptide with strong dendritic cell activity, but lacking neutrophil chemotactic
 30 activity.

APC Chemotaxins Produced by Gene Shuffling Chemokine Polynucleotides

In one embodiment, derivatives of chemokines with enhanced properties (*e.g.*, the ability attract immature dendritic cells but not neutrophils, enhanced chemoattractant activity at lower concentrations, and the like) are constructed using forced *in vitro* genetic evolution using any of several routine techniques, including error-prone PCR or recombination/gene shuffling approaches. Methods for generating new polypeptides with desired activities by gene "shuffling" are known in the art. Various shuffling methods are described in Patten *et al.*, 1997, *Curr. Opin. Biotech.* 8:724-733; Stemmer, 1994, *Nature* 370:389-391; Stemmer *et al.*, 1994, *Proc. Natl. Acad. Sci. USA* 91:10747-10751; Zhao *et al.*, 1997, *Nucleic Acids Res.* 25:1307-1308; Cramer *et al.*, 1998, *Nature* 391:288-291; Cramer *et al.*, 1997, *Nat. Biotech.* 15:436-438; Arnold *et al.*, 1997, *Adv. Biochem. Eng. Biotechnol.* 58:2-14; Zhang *et al.*, 1997, *Proc. Natl. Acad. Sci. USA* 94:4504-4509; Cramer *et al.*, 1996, *Nat. Biotechnol.* 14:315-319; Cramer *et al.*, 1996, *Nat. Med.* 2:100-102; PCT publications WO95/22625; WO97/20078; WO97/35957; WO97/35966; WO98/13487; WO98/13485; PCT 98/00852; PCT 97/24239; and U.S. Patent Nos. 5,605,793, 5,811,238, and 5,928,905. One method of gene shuffling involves conducting a polynucleotide amplification process on overlapping segments of a population of variants of a polynucleotide under conditions whereby one segment serves as a template for extension of another segment, to generate a population of recombinant polynucleotides, and screening or selecting a recombinant polynucleotide or an expression product thereof for a desired property. Some methods of shuffling use random point mutations (typically introduced in a PCR amplification step) as a source of diversity. The resulting polypeptides are tested for chemotactic activity as described above.

In an embodiment, gene shuffling is carried out beginning with a polynucleotide encoding a particular chemokine (*e.g.*, hMCP2, hMCP3, hMIP1 β , hMIP3 α , hMIP3 β , mMIG, mMIP1 γ , mMDC, vMIP1, mC10). In a different embodiment, "family shuffling" is used (see, *e.g.*, Cramer *et al.*, 1998, *Nature* 152:88-91; Chang *et al.*, 1999, *Nat Biotechnol* 17:793-7), and at least two "parental" chemokine-encoding polynucleotides are used in the family shuffling reaction. In an embodiment, at least one of the parental chemokine-encoding polynucleotides

encodes hMIP1 α , hMIP1 α (70aa), mMIP-1 α , hRANTES, hMET-RANTES, mRANTES, hHCC-1, hMPIF-1, hMPIF-1 (22-137), hMPIF-1 (46-137), hMIP-1 δ , hMCP-4, mMCP-5, mMARC, mEotaxin, mMCP-1(JE), mTECK, mMIP-2, mBLC, mMIP-1 γ , mMIG, and mMIP-1 β , hMCP-2, hMCP-3, vMIP-1, hMIP-3 α , hMIP-3 β , mC10, mMDC, hMIP-1 β , vMCK-2, and hLeukotactin or a species homolog thereof.

In a related embodiment, at least two of the parental chemokine-encoding polynucleotides encode a chemokine from the group hMIP1 α , hMIP1 α (70aa), mMIP-1 α , hRANTES, hMET-RANTES, mRANTES, hHCC-1, hMPIF-1, hMPIF-1 (22-137), hMPIF-1 (46-137), hMIP-1 δ , hMCP-4, mMCP-5, mMARC, mEotaxin, mMCP-1(JE), mTECK, mMIP-2, mBLC, mMIP-1 γ , mMIG, and mMIP-1 β , hMCP-2, hMCP-3, vMIP-1, hMIP-3 α , hMIP-3 β , mC10, mMDC, hMIP-1 β , vMCK-2, and hLeukotactin.

In an embodiment, the resulting shuffled ("evolved") molecule comprises at least 10 contiguous residues from at least one, often at least two different naturally occurring chemokines, *e.g.*, such as the sets listed *supra*.

Other APC Chemotaxins Prepared by Mutating of Chemokine Molecules

In other aspects of the invention, a parental APC chemotaxin (*e.g.*, chemokine) is modified by conventional *in vitro* mutagenesis methods (*e.g.*, site directed mutagenesis (Ausubel, *supra*) or *in vitro* genetic manipulation of chemokine-encoding polynucleotides. The activity of the resulting variant can be determined using the *in vitro* and *in vivo* assays described herein.

In another aspect of the invention, a parental APC chemotaxin polypeptide (*e.g.*, a chemokine) is modified (*e.g.*, by *in vitro* genetic manipulation of the chemokine-encoding polynucleotide) to produce an amino- or carboxy-truncated version of the mature protein. Alternatively, these truncated version of the APC chemotaxin can be chemically synthesized or produced by enzymatic processing of a naturally occurring chemokine. In addition, variants with conservative substitutions that retain the desired properties may be used in the methods and compositions of the invention. Conservative substitution tables providing functionally similar amino acids are well known in the art. For example, one exemplary guideline to select conservative substitutions includes (original residue followed by exemplary

substitution): ala/gly or ser; arg/ lys; asn/ gln or his; asp/glu; cys/ser; gln/asn; gly/asp; gly/ala or pro; his/asn or gln; ile/leu or val; leu/ile or val; lys/arg or gln or glu; met/leu or tyr or ile; phe/met or leu or tyr; ser/thr; thr/ser; trp/tyr; tyr/trp or phe; val/ile or leu.

An alternative exemplary guideline uses the following six groups, each
5 containing amino acids that are conservative substitutions for one another:

(1) Alanine (A), Serine (S), Threonine (T)

(2) Aspartic acid (D), Glutamic acid (E)

(3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K)

(5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V), and

10 (6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W); (see also, *e.g.*,
Creighton (1984) *Proteins*, W.H. Freeman and Company; Schulz and Schimer (1979)
Principles of Protein Structure, Springer-Verlag).

Polypeptide Mimetics

Polypeptide mimetics are also suitable for use in the methods of the invention.
The terms “mimetic” and “peptidomimetic” refer to a synthetic chemical compound
that has substantially the same structural and/or functional characteristics as an APC
chemotaxin polypeptide of the invention (*e.g.*, a specific chemokine). The mimetic
can be either entirely composed of synthetic, non-natural analogues of amino acids or
is a chimeric molecule of partly natural peptide amino acids and partly non-natural
analogues of amino acids. The mimetic can also incorporate any amount of natural
amino acid conservative substitutions as long as such substitutions do not
substantially alter the mimetic’s structure and/or activity. Polypeptide mimetic
compositions can contain any combination of nonnatural structural components,
25 which are typically from three structural groups: (a) residue linkage groups other than
the natural amide bond (“peptide bond”) linkages; (b) non-natural residues in place of
naturally occurring amino acid residues; or (c) residues which induce secondary
structural mimicry, *i.e.*, inducing or stabilizing a secondary structure, *e.g.*, a β turn, γ
turn, β sheet, α helix conformation, and the like.

30 A polypeptide can be characterized as a mimetic when all or some of its
residues are joined by chemical means other than natural peptide bonds. Individual
peptidomimetic residues can be joined by peptide bonds, other chemical bonds or

coupling means, such as, *e.g.*, glutaraldehyde, N-hydroxysuccinimide esters, bifunctional maleimides, N,N'-dicyclohexylcarbodiimide (DCC) or N,N'-diisopropylcarbodiimide (DIC). Linking groups that can be an alternative to the traditional amide bond ("peptide bond") linkages include, *e.g.*, ketomethylene (*e.g.*, -C(=O)-CH₂- for -C(=O)-NH-), aminomethylene (CH₂-NH), ethylene, olefin (CH=CH), ether (CH₂-O), thioether (CH₂-S), tetrazole (CN₄-), thiazole, retroamide, thioamide, or ester (see, *e.g.*, Spatola (1983) in *Chemistry and Biochemistry of Amino Acids, Peptides and Proteins*, Vol. 7, pp 267-357, "Peptide Backbone Modifications," Marcell Dekker, NY).

A polypeptide can also be characterized as a mimetic by containing all or some non-natural residues in place of naturally occurring amino acid residues. Nonnatural residues are well described in the scientific and patent literature; a few exemplary nonnatural compositions useful as mimetics of natural amino acid residues and guidelines are described below.

Mimetics of aromatic amino acids can be generated by replacing by, *e.g.*, D- or L- naphylalanine; D- or L- phenylglycine; D- or L-2 thieneylalanine; D- or L-1, -2, 3- or 4- pyreneylalanine; D- or L-3 thieneylalanine; D- or L-(2-pyridinyl)-alanine; D- or L-(3-pyridinyl)-alanine; D- or L-(2-pyrazinyl)-alanine; D- or L-(4-isopropyl)-phenylglycine; D-(trifluoromethyl)-phenylglycine; D-(trifluoromethyl)-phenylalanine; D-p-fluorophenylalanine; D- or L-p-biphenylphenylalanine; K- or L-p-methoxybiphenylphenylalanine; D- or L-2-indole(alkyl)alanines; and, D- or L-alkylainines, where alkyl can be substituted or unsubstituted methyl, ethyl, propyl, hexyl, butyl, pentyl, isopropyl, iso-butyl, sec-isotyl, iso-pentyl, or a non-acidic amino acids. Aromatic rings of a nonnatural amino acid include, *e.g.*, thiazolyl, thiophenyl, pyrazolyl, benzimidazolyl, naphthyl, furanyl, pyrrolyl, and pyridyl aromatic rings.

Mimetics of acidic amino acids can be generated by substitution by, *e.g.*, non-carboxylate amino acids while maintaining a negative charge; (phosphono)alanine; sulfated threonine. Carboxyl side groups (*e.g.*, aspartyl or glutamyl) can also be selectively modified by reaction with carbodiimides (R'-N-C-N-R') such as, *e.g.*, 1-cyclohexyl-3(2-morpholinyl-(4-ethyl) carbodiimide or 1-ethyl-3(4-azonia- 4,4-dimetholpentyl) carbodiimide. Aspartyl or glutamyl can also be converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

Mimetics of basic amino acids can be generated by substitution with, *e.g.*, (in addition to lysine and arginine) the amino acids ornithine, citrulline, or (guanidino)-acetic acid, or (guanidino)alkyl-acetic acid, where alkyl is defined above. Nitrile derivative (*e.g.*, containing the CN-moiety in place of COOH) can be substituted for asparagine or glutamine. Asparaginy and glutaminy residues can be deaminated to the corresponding aspartyl or glutamyl residues.

Arginine residue mimetics can be generated by reacting arginyl with, *e.g.*, one or more conventional reagents, including, *e.g.*, phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, or ninhydrin, preferably under alkaline conditions.

Tyrosine residue mimetics can be generated by reacting tyrosyl with, *e.g.*, aromatic diazonium compounds or tetranitromethane. N-acetylimidazol and tetranitromethane can be used to form O-acetyl tyrosyl species and 3-nitro derivatives, respectively.

Cysteine residue mimetics can be generated by reacting cysteinyl residues with, *e.g.*, α -haloacetates such as 2-chloroacetic acid or chloroacetamide and corresponding amines; to give carboxymethyl or carboxyamidomethyl derivatives. Cysteine residue mimetics can also be generated by reacting cysteinyl residues with, *e.g.*, bromo-trifluoroacetone, α -bromo- β -(5-imidazolyl) propionic acid; chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide; methyl 2-pyridyl disulfide; p-chloromercuribenzoate; 2-chloromercuri-4 nitrophenol; or, chloro-7-nitrobenzo-oxa-1,3-diazole.

Lysine mimetics can be generated (and amino terminal residues can be altered) by reacting lysinyl with, *e.g.*, succinic or other carboxylic acid anhydrides. Lysine and other α -amino-containing residue mimetics can also be generated by reaction with imidoesters, such as methyl picolinimidate, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitrobenzenesulfonic acid, O-methylisourea, 2,4-pentanedione, and transamidase-catalyzed reactions with glyoxylate.

Mimetics of methionine can be generated by reaction with, *e.g.*, methionine sulfoxide. Mimetics of proline include, *e.g.*, pipecolic acid, thiazolidine carboxylic acid, 3- or 4- hydroxy proline, dehydropoline, 3- or 4-methylproline, or 3,3,-dimethylproline. Histidine residue mimetics can be generated by reacting histidyl with, *e.g.*, diethylprocarbonate or para-bromophenacyl bromide.

Other mimetics include, *e.g.*, those generated by hydroxylation of proline and lysine; phosphorylation of the hydroxyl groups of seryl or threonyl residues; methylation of the α -amino groups of lysine, arginine and histidine; acetylation of the N-terminal amine; methylation of main chain amide residues or substitution with N-methyl amino acids; or amidation of C-terminal carboxyl groups. A component of a natural polypeptide can also be replaced by an amino acid (or peptidomimetic residue) of the opposite chirality. Thus, any amino acid naturally occurring in the L-configuration (also referred to as the R or S, depending upon the structure of the chemical entity) can be replaced with the amino acid of the same chemical structural type or a peptidomimetic, but of the opposite chirality, generally referred to as the D-amino acid, but which can additionally be referred to as the R- or S- form.

The mimetics of the invention can also include compositions that contain a structural mimetic residue, particularly a residue that induces or mimics secondary structures, such as a β turn, β sheet, α helix structures, γ turns, and the like. For example, substitution of natural amino acid residues with D-amino acids; N- α -methyl amino acids; C- α -methyl amino acids; or dehydroamino acids within a peptide can induce or stabilize β turns, γ turns, β sheets or α helix conformations. β turn mimetic structures have been described, *e.g.*, by Nagai (1985) *Tet. Lett.* 26:647-650; Feigl (1986) *J. Amer. Chem. Soc.* 108:181-182; Kahn (1988) *J. Amer. Chem. Soc.* 110:1638-1639; Kemp (1988) *Tet. Lett.* 29:5057-5060; Kahn (1988) *J. Molec. Recognition* 1:75-79. β sheet mimetic structures have been described, *e.g.*, by Smith (1992) *J. Amer. Chem. Soc.* 114:10672-10674. For example, a type VI β turn induced by a *cis* amide surrogate, 1,5-disubstituted tetrazol, is described by Beusen (1995) *Biopolymers* 36:181-200. Incorporation of achiral omega-amino acid residues to generate polymethylene units as a substitution for amide bonds is described by Banerjee (1996) *Biopolymers* 39:769-777. Secondary structures of polypeptides can be analyzed by, *e.g.*, high-field ^1H NMR or 2D NMR spectroscopy; see, *e.g.*, Higgins (1997) *J. Pept. Res.* 50:421-435. See also, Hruby (1997) *Biopolymers* 43:219-266, Balaji, *et al.*, U.S. Pat. No. 5,612,895.

Specific examples of mimetics which can be incorporated into the polypeptides of the invention include those described by, *e.g.*, Zhang (1998) *Biochemistry* 37:12465-12476, who constructed functionally active (R and

S)- γ -lactam conformational mimetics using a 3-(R or S)-amino- 2-oxo-1- pyrrolidine-acetamido moiety in place of the Pro-Gly of the tridecapeptide *Saccharomyces cerevisiae* α -factor mating pheromone. Brady (1998) *J. Med. Chem.* 41:401-406, used a resin-based route for the synthesis of thrombin inhibitor mimetic with a range of lipophilic carboxylic amides. Baures (1997, *J. Med. Chem.* 40:3594-3600) constructed a diketopiperazine conformational mimic into a L-prolyl-L-leucylglycinamide structure and into the bicyclic lactam PLG peptidomimetic structure of dopamine receptor. Beaulieu (1997, *J. Med. Chem.* 40:2164-2176) used a (hydroxyethyl)amidosuccinoyl core to synthesize a peptidomimetic structure to inhibit HIV viral protease activity. Misicka (1997, *J. Pept. Res.* 50:48-54) designed mimetics of deltorphin I and dermenkephalin containing stereoisomer of the unusual amino acid beta-methylphenylalanine to generate peptides with increased ligand binding specificity.

The skilled artisan will recognize that individual synthetic residues and polypeptides incorporating mimetics can be synthesized using a variety of procedures and methodologies that are well described in the scientific and patent literature, *e.g.*, *Organic Syntheses Collective Volumes*, Gilman *et al.* (Eds) John Wiley & Sons, Inc., NY. Polypeptides incorporating mimetics can also be made using solid phase synthetic procedures as described, *e.g.*, by Di Marchi, *et al.*, and U.S. Pat. No. 5,422,426. Mimetics of the invention can also be synthesized using combinatorial methodologies. Various techniques for generation of peptide and peptidomimetic libraries are well known, and include, *e.g.*, multipin, tea bag, and split-couple-mix techniques; see, *e.g.*, al-Obeidi (1998) *Mol. Biotechnol.* 9:205-223; Hruby (1997) *Curr. Opin. Chem. Biol.* 1:114-119; Ostergaard (1997) *Mol. Divers.* 3:17-27; and Ostresh (1996) *Methods Enzymol.* 267:220-234.

Small Chemokine Mimetics

In an embodiment, small molecule chemokine mimetics are used to mobilize APCs. Typically, small molecule mimetics are identified using high throughput screening technologies for small molecule compounds that bind chemokine receptors (CRs) and transduce signal (*e.g.*, calcium ion mobilization or other chemokine receptor-mediated responses). For example, small molecules can be screened initially

for the ability to agonize chemokine receptors that are expressed on immature dendritic cells and/or are not expressed on other cells (see, *e.g.*, Table 2B). The agonizing activity can be detected in a variety of ways, such as detecting Ca²⁺ mobilization responses on transfectant cells expressing cloned chemokine receptors known to be present on APCs.

The chemotactic properties of the molecules are then determined using the assays described *supra*, and those with desired specificity (*e.g.*, chemotactic for immature but not mature dendritic cells) are used in the methods of the present invention.

Chemotactic Compositions

The chemotactic compositions of the invention contain one or more APC chemotaxins or chemotaxin-encoding polynucleotides. In an embodiment, the composition contains an APC chemotaxin that is an isolated or recombinant polynucleotide or polypeptide. In an embodiment, the APC chemotaxin(s) is/are the predominant species (*i.e.*, greater than about 50%, more often greater than about 80% by weight of the total of the members of the class of molecule in the composition) of its class (*e.g.*, polypeptide, polynucleotide, lipid, carbohydrate) in the composition. In other embodiments, the APC chemotaxin(s) is "biologically pure." The words "isolated," "pure" "substantially purified" or "biologically pure" refer to material that is substantially or essentially free from components which normally accompany it as found in its native state. Thus, in an embodiment, the chemotactic compositions of the invention contain APC chemotaxins free of materials normally associated with their *in situ* environment (if naturally occurring). Typically, the isolated, chemotaxins of the invention are at least about 80% pure, usually at least about 90%, and preferably at least about 95%. Protein purity or homogeneity may be determined by a number of means well known in the art, such as polyacrylamide gel electrophoresis of a protein sample, followed by visualization upon staining. For certain purposes high resolution will be needed and HPLC or a similar means for purification utilized.

In embodiments, the compositions may additionally contain an excipient or carrier, such as described *infra*. In some embodiments of the invention, the

composition includes one or more antigens (*i.e.*, the antigen to which it is desired to induce or enhance an immune response), as is discussed in greater detail *infra*.

In embodiments, the compositions may contain a conventional adjuvant. Conventional adjuvants typically convert soluble protein antigens into particulate material and often include bacteria or bacterial products. Exemplary conventional adjuvants include Freund's incomplete adjuvant, Freund's complete adjuvant, Merck Adjuvant 65, AS-2, alum, aluminum phosphate, mineral gels such as aluminum hydroxide, and surface active substances such as lysolecithin, pluronic polyols, polyanions, peptides, oil emulsions, keyhole limpet hemocyanin, and dinitrophenol. Other useful adjuvants include, but are not limited to, bacterial capsular polysaccharides, dextran, IL-12, GM-CSF, CD40 ligand, IFN- γ , IL-1, IL-2, IL-3, IL-4, IL-10, IL-13, IL-18 or any cytokine.

In one aspect, the invention provides a method of formulating a composition capable of inducing an immune response (that is, the composition possesses the characteristic of being able to elicit an immune response if administered) to a specified antigen in a subject by identifying a polypeptide having the activity of an antigen-presenting cell chemotaxin (APC chemotaxin) as described herein and combining the polypeptide with the antigen. In an embodiment, a pharmaceutically acceptable excipient is also included.

Antigens

In one aspect, the present invention provides a method of eliciting or enhancing an immune response to an antigen, *e.g.*, a predetermined or specified antigen. An antigen is a molecule that reacts with an antibody. In some embodiments the antigen is an immunogen. In some embodiments the antigen is linked to a protein carrier. For example, in one embodiment of the invention, an APC chemotaxin and an antigen are physically linked (*e.g.*, made as a fusion protein, stably cross-linked using chemical cross-linkers, or linked via complexes such as biotin and streptavidin).

An antigen is typically a peptide, a polypeptide, chemical compound, microbial pathogen, bacteria (*e.g.*, live, attenuated, or inactivated), a virus (including inactivated virus particles, modified live viral particles, and recombinant virus particles), a recombinant cell, glycoproteins, lipoproteins, glycopeptides, lipopeptides,

toxoids, carbohydrates, tumor-specific antigens, and other immunogenic components of pathogens. In one embodiment, mixtures of two or more antigens are employed. In some embodiments, the antigen is biologically pure.

In one embodiment, the methods and reagents of the invention are used to provide protection from exogenous foreign infectious pathogenic agents (such as bacteria, virus, and the like) prior to expected or possible exposure. In a related embodiment, the methods and reagents of the invention are used to provide therapeutic effects against exogenous foreign pathogens to which an individual has been exposed or to an individual displaying symptoms of exposure.

In one embodiment, reagents and methods of the invention are used to treat cancers, including, but not limited to, melanomas, lung cancers, thyroid carcinomas, breast cancers, renal cell carcinomas, squamous cell carcinomas, brain tumors and skin cancers. In one embodiment, the antigen is a tumor-associated antigen (tumor specific-antigen). Tumor antigens are molecules, especially cell surface proteins, which are differentially expressed in tumor cells relative to non-tumor tissues (*e.g.*, telomerase).

For prophylactic use, compositions containing APC chemotaxins are administered (*e.g.*, in conjunction with antigens) to a subject susceptible to or otherwise at risk of a disease, *e.g.*, a tumor, cancer, infection, and the like. For therapeutic use, compositions containing the APC chemotaxins are administered (*e.g.*, in conjunction with antigens) to a subject once a disease, *e.g.* a tumor, cancer, infection, and the like, is detected or diagnosed, or after surgical removal, *e.g.*, of tumors.

Exemplary antigens or vaccine components of the invention include antigens derived from microbial pathogens such as bacteria [*e.g.*, Pertussis (*Bordetella pertussis*, inactivated whole organism); Cholera (*Vibrio cholerae*, whole killed organism); Meningitis (*Neisseria meningitidis*, polysaccharide from organism); Lyme Disease (*Borrelia burgdorferi*, lipoprotein OspA); Haemophilus B (*Haemophilus influenza B* polysaccharide, Tetanus conjugate or OmpC); Pneumonia (*Streptococcus pneumoniae* capsular polysaccharide) Typhoid (*Salmonella typhi* polysaccharide vaccine, killed whole organism)], viruses including inactivated virus particles, modified live viral particles, and recombinant virus particles to Influenza virus;

Hepatitis A; Hepatitis B; Measles; Rubella virus; Mumps ; Rabies; Poliovirus; Japanese Encephalitis virus; Rotavirus; Varicella], Diphtheria (*Corynebacterium diphtheriae*) and Tetanus (*Clostridium tetani*).

5 *Polynucleotide Chemotactic Compositions*

In one aspect, the APC chemotaxin, the antigen, or both are delivered as DNA such that the polypeptides are generated *in situ*. In one embodiment, the DNA is “naked,” as described, for example, in Ulmer *et al.*, *Science* 259:1745-1749, 1993 and reviewed by Cohen, 1993, *Science* 259:1691-1692. The uptake of naked DNA may
10 be increased by coating the DNA onto a carrier, *e.g.* biodegradable beads, which is efficiently transported into the cells. In such vaccines, the DNA may be present within any of a variety of delivery systems known to those of ordinary skill in the art, including nucleic acid expression systems, bacterial and viral expression systems. Techniques for incorporating DNA into such expression systems are well known to those of ordinary skill in the art. See, *e.g.*, WO90/11092, WO93/24640, WO
15 93/17706, and U.S. Pat. No. 5,736,524.

Vectors, used to shuttle genetic material from organism to organism, can be divided into two general classes: Cloning vectors are replicating plasmid or phage with regions that are non-essential for propagation in an appropriate host cell and into which foreign DNA can be inserted; the foreign DNA is replicated and propagated as if it were a component of the vector. An expression vector (such as a plasmid, yeast,
20 or animal virus genome) is used to introduce foreign genetic material into a host cell or tissue in order to transcribe and translate the foreign DNA, such as APC chemotaxin. In expression vectors, the introduced DNA is operably-linked to elements such as promoters that signal to the host cell to transcribe the inserted DNA. Some promoters are exceptionally useful, such as inducible promoters that control gene transcription in response to specific factors. Operably-linking an APC chemotaxin and/or antigen polynucleotide to an inducible promoter can control the expression of an APC chemotaxin and/or antigen polypeptide or fragments.
25 Examples of classic inducible promoters include those that are responsive to α -interferon, heat shock, heavy metal ions, and steroids such as glucocorticoids (Kaufman, 1990. *Methods Enzymol* 185: 487-511.) and tetracycline. Other desirable
30

inducible promoters include those that are not endogenous to the cells in which the construct is being introduced, but, however, are responsive in those cells when the induction agent is exogenously supplied. In general, useful expression vectors are often plasmids. However, other forms of expression vectors, such as viral vectors (e.g., replication defective retroviruses, adenoviruses and adeno-associated viruses) are contemplated.

Vector choice is dictated by the organism or cells being used and the desired fate of the vector. Vectors may replicate once in the target cells, or may be “suicide” vectors. In general, vectors comprise signal sequences, origins of replication, marker genes, enhancer elements, promoters, and transcription termination sequences.

Administration of APC Chemotaxin and Antigen

In embodiments, the compositions can contain one or more antigens (or antigen-encoding polynucleotides). The antigens can be administered in combination with the APC chemotaxin (*i.e.*, in the same mixture). Alternatively, they can be administered separately.

In one aspect, the invention provides an immunization method in which a combination of one or more antigens (or antigen-encoding polynucleotides) and one or more APC chemotaxins (or an APC chemotaxin-encoding polynucleotides) are administered to a subject. Optionally, the antigen or APC chemotaxin is administered in a delivery vehicle such as a physiologically acceptable excipient.

Administration and Dose Scheduling

In one embodiment of the invention, an antigen is administered simultaneously with the chemotactic composition. In an alternative embodiment, the antigen and the chemotactic composition are administered at different times, typically to the same site. For example, the chemotactic composition (without the antigen) can be administered between about 15 minutes and about 96 hours prior to the administration of the antigen, more often between about 15 minutes and about 48 hours, more often between 24 hours and 96 hours, often between about 48 hours and 72 hours or between 72 hours and 96 hours prior to the administration of the antigen.

When the chemotactic composition and an antigen composition are injected at the same site in a subject, preferably the injections are within 2 cm of each other, preferably within 1 cm or preferably within 0.5 cm of each other on the two dimensional surface of the body. In this embodiment, the administrations should also
5 be done to a similar depth and to the same tissue layer (*i.e.*, both injections should be subcutaneous or both intradermal). For intramuscular injections, the depth should be more precisely monitored to achieve a three dimensional equivalent placement of the APC chemotaxin and the antigen to within 2 cm of each other, preferably to within 1 cm, and more preferably to within 0.5 cm. This is easily accomplished by physicians,
10 nurses and other medically trained personal. The injection site can be marked with an indelible ink to assist the physician.

In one embodiment, only one dose (administration) of the composition is given. In another embodiment, the first administration is followed by boosting doses. In an embodiment, the APC chemotaxin is administered in multiple doses, often in
15 combination with an antigen (*e.g.*, by co-administration). In various embodiments, the APC chemotaxin composition (optionally including antigen) is administered once, twice, three times, or more than three times. The number of doses administered to a subject is dependent upon the antigen, the extent of the disease, and the response of a subject to the chemotactic composition. It will be appreciated that it is within the
20 scope of the present invention that a suitable number of doses includes any number required to immunize an animal (*i.e.*, to a predetermined antigen).

In one embodiment of the invention, a second administration (booster) of the chemotactic composition and antigen occurs between about 7 days and 1 year after the original administration. In an embodiment, a second administration (booster) of
25 the chemotactic composition and antigen occurs between about 14 days and 6 months after the original administration. Alternatively, a second administration (booster) of the chemotactic composition and antigen occurs between about 21 days and 3 months after the original administration, often between about 28 days and 2 months after the original administration. In one embodiment third administration (second booster)
30 occurs between about 14 days and 10 years after the original administration, *e.g.*, between about 14 days and 3 years after the original administration, often between about 21 days and 1 year after the original administration, very often between about

28 days and 6 months after the original administration. Subsequent boosters may be administered at 2 week intervals, or 1 month, 3 month or 6 month to 10 year intervals.

It will be recognized by those of ordinary skill that a variety of vaccine administration doses and schedules can be developed based upon the parameters discussed above and known in the art, and that determination of an effective amount and number of doses of chemotaxins of the invention, antigens, or some combination of chemotaxin(s) and antigen(s) for administration is well within the capabilities of those skilled in the art.

Effective Dose

Typically, the amount of APC chemotaxin and antigen will be administered to a subject that is sufficient to immunize an animal against an antigen (*i.e.*, an “immunologically effective dose” or a “therapeutically effective dose”). An amount adequate to accomplish an “immunologically effective dose” will depend on, *e.g.*, the APC chemotaxin and antigen composition, the manner of administration, the stage and severity of the disease being treated, the weight and general state of health of the subject, and the judgment of the prescribing physician.

The effective dose of antigen and APC chemotaxin can be formulated in animal models to achieve an induction of an immune response using techniques that are well known in the art. One having ordinary skill in the art can readily optimize administration to humans based on animal data. When the APC chemotaxin is a polypeptide, such as a chemokine, a dose will typically be between about 1 fg and about 100 μ g, often between about 1 pg and about 100 μ g, more often between about 1 ng and about 50 μ g, and usually between about 100 ng and about 50 μ g. In some embodiments, the dose is between about 1 fg and about 100 μ g per kg subject body weight, often between about 1 pg and about 100 μ g, more often between about 1 ng and about 50 μ g, and usually between about 100 ng and about 50 μ g per kg subject body weight.

The amount of antigen that is administered will vary with the identity and characteristics of the antigen. In one embodiment, a chemotactic composition of the present invention contains one or more antigens and one or more chemotaxins at a molar or weight ratio of about 1:1000 or greater, chemotaxin to antigen. In another

embodiment, the ratio of chemotaxin to antigen in the composition is between about 1:10 and 1:1000. In another embodiment, the ratio of antigen to chemotaxin in the composition is between about 1:10 and 1:1000, or greater than 1:1000 or greater. In another embodiment, the ratio of antigen to chemotaxin in the composition is between about 1:10 and 10:1.

Carriers, Excipients, Conventional Adjuvants, Mode of Administration

The APC chemotaxin-containing compositions of the invention may be administered in a variety of ways, as described herein. In various embodiments, the chemotactic composition includes carriers and excipients (including but not limited to buffers, carbohydrates, mannitol, proteins, polypeptides or amino acids such as glycine, antioxidants, bacteriostats, chelating agents, suspending agents, thickening agents and/or preservatives), water, oils, saline solutions, aqueous dextrose and glycerol solutions, other pharmaceutically acceptable auxiliary substances as required to approximate physiological conditions, such as buffering agents, tonicity adjusting agents, wetting agents and the like, and/or a conventional adjuvant (*e.g.*, as discussed *supra*). It will be recognized that, while any suitable carrier known to those of ordinary skill in the art may be employed to administer the compositions of this invention, the type of carrier will vary depending on the mode of administration. Compounds may also be encapsulated within liposomes using well-known technology. Biodegradable microspheres may also be employed as carriers for the pharmaceutical compositions of this invention. Suitable biodegradable microspheres are disclosed, for example, in U.S. Patent Nos. 4,897,268; 5,075,109; 5,928,647; 5,811,128; 5,820,883; 5,853,763; 5,814,344 and 5,942,252.

The compositions of the invention may be sterilized by conventional, well-known sterilization techniques, or may be sterile filtered. The resulting aqueous solutions may be packaged for use as is, or lyophilized, the lyophilized preparation being combined with a sterile solution prior to administration.

The APC chemotaxin composition of the invention may be administered in a variety of ways, including by injection (*e.g.*, intradermal, subcutaneous, intramuscular, intraperitoneal and the like), by inhalation, by topical administration, by suppository, by using a transdermal patch or by mouth.

When administration is by injection, the chemotaxin(s) may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hanks solution, Ringer's solution, or physiological saline buffer. The solution may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

- 5 Alternatively, the chemotactic composition may be in powder form for constitution with a suitable vehicle, *e.g.*, sterile pyrogen-free water, before use.

When administration is by inhalation, the chemotaxin(s) may be delivered in the form of an aerosol spray from pressurized packs or a nebulizer with the use of a suitable propellant, *e.g.*, dichlorodifluoromethane, trichlorofluoromethane, carbon
10 dioxide or other suitable gas. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, *e.g.*, gelatin for use in an inhaler or insufflator may be formulated containing a powder mix of the proteins and a suitable powder base such as lactose or starch.

15 When administration is by topical administration, the chemotactic composition may be formulated as solutions, gels, ointments, creams, suspensions, and the like, as are well known in the art. In some embodiments, administration is by means of a transdermal patch.

When administration is by suppository (*e.g.*, rectal or vaginal), compositions
20 may also be formulated in compositions containing conventional suppository bases.

When administration is oral, a composition can be readily formulated by combining the chemotaxin with pharmaceutically acceptable carriers well known in the art. A solid carrier, such as mannitol, lactose, magnesium stearate, and the like may be employed; such carriers enable the chemotaxin to be formulated as tablets,
25 pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions and the like, for oral ingestion by a subject to be treated. For oral solid formulations such as, for example, powders, capsules and tablets, suitable excipients include fillers such as sugars, cellulose preparation, granulating agents, and binding agents.

30 Nucleic acid molecules, such as those encoding APC chemotaxins, can be inserted into vectors and used as gene therapy vectors. Gene therapy vectors can be delivered to a subject by, for example, intravenous injection, local administration (Nabel et al., US Patent No. 5,328,470 1994. USA), or by stereotactic injection

(Chen et al., 1994. Proc Natl Acad Sci U S A 91: 3054-7.). The pharmaceutical preparation of a gene therapy vector can include an acceptable diluent or can comprise a slow release matrix in which the gene delivery vehicle is imbedded. Alternatively, where the complete gene delivery vector can be produced intact from recombinant cells, *e.g.*, retroviral vectors, the pharmaceutical preparation can include one or more cells that produce the gene delivery system.

Other convenient carriers, as well-known in the art, also include multivalent carriers, such as bacterial capsular polysaccharide, a dextran or a genetically engineered vector. In addition, sustained-release formulations the include, for example, APC chemotaxin molecules and/or antigens, allow for the release of APC chemotaxin over extended periods of time, such that without the sustained release formulation, the APC chemotaxin would be cleared from a subject's system, and/or degraded by, for example, proteases and simple hydrolysis before eliciting or enhancing an immune response.

Vaccination for Monoclonal and Polyclonal Antibody Production

Methods of producing polyclonal and monoclonal antibodies, including binding fragments (*e.g.*, $F_{(ab)2}$) and single chain versions, are well known to those of skill in the art. *See, e.g.*, Coligan, 1991, *Current Protocols in Immunology*, Wiley/Greene, NY; and Harlow and Lane, 1989, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Press, NY. However, many antigens are not capable of triggering an adequate antibody response in animals. In one embodiment, a composition comprising a chemotaxin of the invention and an antigen is administered to an animal as described herein, thus inducing or enhancing the immune response in the animal. Polyclonal or monoclonal antibodies are subsequently prepared by standard techniques.

Stimulation of Innate Immune Response

In another aspect of the invention, the compositions of the invention are administered to a subject to stimulate the innate immune response. The innate immune response is body's initial defense against pathogens and is elicited by a variety of cells including APCs. These cells express surface and cytoplasmic

receptors that recognize molecules of foreign origin (*e.g.*, bacterial and viral nucleic acids, proteins, carbohydrates). Upon detecting these signals, the dendritic cells and macrophage elicit a defensive response that includes the release of cytokines (including interferons, TNF- α , and IL-12) and chemokines that attract cells such as

5 immature dendritic cells, macrophage, NK cells, and granulocytes, to the site of challenge.

The compositions of the invention are useful, not only to attract dendritic cells and other cells to the site of administration, but also to stimulate these cells into eliciting elements of the innate immune response to confer non-specific protection

10 while the body is generating the adaptive response.

In one embodiment, a composition of the invention is administered (without antigen) prior or post exposure of an anticipated infection. In another embodiment, the chemokine is administered with “foreign” molecules (*e.g.*, bacterial or viral nucleic acids, proteins, carbohydrates, or synthetic elements which mimic these

15 elements).

Kits

In an aspect, the invention provides kits containing one or more of the following in a package or container: (1) a chemotactic composition of the invention;

20 (2) a pharmaceutically acceptable adjuvant or excipient; (3) an antigen (*e.g.*, a biologically pure antigen); (4) a vehicle for administration, such as a syringe; (5) instructions for administration. Embodiments in which two or more of components (1) - (5) are found in the same container are also contemplated.

When a kit is supplied, the different components of the composition may be

25 packaged in separate containers and admixed immediately before use. Such packaging of the components separately may permit long-term storage without losing the active components' functions.

(a) *Containers, packages and vessels*

The reagents included in the kits can be supplied in containers of any sort such

30 that the life of the different components are preserved and are not adsorbed or altered by the materials of the container. For example, sealed glass ampules may contain lyophilized APC chemotaxin polypeptide or polynucleotide, or buffers that have been

packaged under a neutral, non-reacting gas, such as nitrogen. Ampules may consist of any suitable material, such as glass, organic polymers, such as polycarbonate, polystyrene, *etc.*; ceramic, metal or any other material typically employed to hold similar reagents. Other examples of suitable containers include simple bottles that may be fabricated from similar substances as ampules, and envelopes, that may comprise foil-lined interiors, such as aluminum or an alloy. Other containers include test tubes, vials, flasks, bottles, syringes, or the like. Containers may have a sterile access port, such as a bottle having a stopper that can be pierced by a hypodermic injection needle. Other containers may have two compartments that are separated by a readily removable membrane that upon removal permits the components to be mixed. Removable membranes may be glass, plastic, rubber, *etc.*

(b) *Instructional materials*

Kits may also be supplied with instructional materials. Instructions may be printed on paper or other substrate, and/or may be supplied as an electronic-readable medium, such as a floppy disc, CD-ROM, DVD-ROM, Zip disc, videotape, audiotape, *etc.* Detailed instructions may not be physically associated with the kit; instead, a user may be directed to an internet web site specified by the manufacturer or distributor of the kit, or supplied as electronic mail.

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25

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EXAMPLES

Example 1

Chemotaxis assays were carried out using purified cells and a 96-well chemotaxis microchamber (ChemoTx[®], NeuroProbes, Inc., Gaithersburg, MD). In this assay, a porous polycarbonate filter was used to allow formation of a chemoattractant concentration gradient across the filter, as well as to allow cells to migrate into the filter or through into the lower well.

To perform an immature dendritic cell chemotaxis assay, 29 μ l of a candidate or known APC chemotaxin at 0, 1, 10 and 100 nM CHECK concentrations were placed in the wells of the lower chamber. The filter was placed on the top of the chamber, so that the chemoattractant solution touched the under side of the filter. Day 7 immature dendritic cells were harvested, washed once with chemotaxis buffer containing 0.1% BSA (Sigma) in HBSS (Life Technologies), with Ca^{++} and Mg^{++} , and finally resuspended in chemotaxis buffer at 5×10^6 per ml. Twenty microliters of cells were carefully placed onto the filter. Migration was allowed to proceed for 90 minutes at 37°C in a tissue culture incubator. Migration was terminated by removing non-migrating cells on the top of the filter using a rubber scraper. The filter was removed from the apparatus and rinsed with DPBS. (The lower chamber was inspected microscopically to determine if any cells had migrated into the wells. If a significant number of cells were present in the wells, quantification was done in the wells as well as the filter). To detect those cells that had migrated into the polycarbonate filter in the immature dendritic cell migration assay, the filter, after being removed from the apparatus and rinsed with DPBS (Hyclone), was stained with a cell staining solution such as the Hema3 staining kit (Fisher Scientific). The filter was immersed into the three separate solutions sequentially, each for approximately 5 seconds. After the last solution, the filter was rinsed in water several times. The filter was allowed to air dry and the signal was determined by reading the filter on a plate reader (Molecular Devices) at wavelength 540 nm. The magnitude of migration was calculated as the ratio of absorbance between the wells with chemoattractants and the wells with chemotaxis buffer alone.

Example 2Chemokine Injection Into Mice

This example describes an *in vivo* assay in which the ability of several chemokines to attract dendritic cells is demonstrated.

The following chemokines were obtained from R&D Systems (Minneapolis, MN): MCP2, MCP3, MIP1 β , MIP3 α , MIP3 β , RANTES, mMIG, mMDC, mC10, vMIP1. Each chemokine (2 μ g in PBS) was injected intradermally into a different BALB/c mouse. One mouse received a control injection of PBS with no chemokine. Seventy-two hours after injection, the mice were euthanized, and the area around the injection site was excised and subjected to immunohistology. Frozen sections were stained with anti-DEC-205 antibody (available from Bio-Whittaker Molecular Applications, Rockland, ME; Kraal *et al.*, 1986, *J. Exp. Med.* 163:981), which recognizes a surface molecule specific to dendritic cells. A relative staining number on a scale of 0 to 5 was assigned to each section (0 = lowest infiltration, 5 = highest infiltration). Results are shown in Table 4.

Several chemokines elicited a DEC-205+ (dendritic cell) infiltration to the site of injection after 72 hours when injected intradermally. Of these MCP3, MIP3 β , mMIG, mMDC, mC10, and viral MIP1 showed DC infiltration; MCP3, mMDC and mC10 showed the best infiltration.

Table 4 Dendritic Cell Infiltration *in vivo* Assay

Chemokine	DC Infiltration
MCP2	0
MCP3	3
MIP1b	0
MIP3a	0
MIP3 β	1
RANTES	0
mMIG	2
mMDC	3
mC10	4
vMIP1	0
PBS	0

Example 3Chemokine Injection Into Rhesus monkeys, Experiment 1

This example demonstrates that certain chemokines injected intradermally to a non-human primate elicit mononuclear infiltration to the site of injection.

Coded, sterile chemokines (2 μ g in PBS) were injected intradermally (100 μ l injection volume) into the upper arm of Rhesus macaques under anesthesia. In each case, two monkeys were each given two injections of the same chemokine at two different locations (*e.g.* left arm versus right arm). After 72 hours, one injection site was punch-biopsied and separated into an edge and a center sample and both samples were prepared for immunohistology. After 96 hours, the other injection site was punch-biopsied and separated into an edge and a center sample, and both samples were prepared for immunohistology. The results are presented in Table 5, wherein each row represents a single animal. A section of the prepared tissue was stained with hematoxylin and eosin, and mononuclear cells were identified by morphology (*e.g.*, being mononuclear in contrast to polynuclear). GM-CSF (200 μ g doses) and RANTES (20 μ g doses) were used as positive controls. Injection of PBS and analysis of non-injected tissues were used as negative controls.

Mononuclear cell infiltration was score based on the following scale of 0 to 5:
 0= a very mild perivascular mononuclear inflammatory infiltrate seen throughout the dermis; 1= a mild perivascular mononuclear inflammatory infiltrate seen throughout the dermis; 2= a mild/moderate perivascular mononuclear inflammatory infiltrate seen throughout the dermis; 3= a moderate perivascular mononuclear inflammatory infiltrate seen throughout the dermis; 4= an extensive perivascular mononuclear inflammatory infiltrate seen throughout the dermis; 5= a florid perivascular mononuclear inflammatory infiltrate seen throughout the dermis. Intermediate scores are indicated, *e.g.*, “2/3” represents a score between 2 and 3.

GM-CSF (200 µg dose) showed between a 2 and 3 level of infiltration at 72 hours, as did RANTES (20ug). Additional internally coded samples of RANTES (20 µg dose) showed up to a 2 level of infiltration. Negative controls (PBS) usually had a 0 level of infiltrate

Of the chemokines tested, MCP-2, MCP-3, MIP-1β, MIP3α, MIP3β, RANTES, mMIG, mMDC, mC10, and viral MIP1 showed mononuclear infiltration, with mMDC, mC10, and viral MIP1 showing the highest level of infiltration. In general, greater infiltration was apparent after 72 hours than after 96 hours.

Table 5 Mononuclear Cell Infiltration

Chemokine	72 h		96 h	
	Center	Edge	Center	Edge
GM-CSF (200 ug)*	0	0	3	2/3
RANTES (20 ug)*	0/1	1	2/3	1 / 2
MCP2	1/2	1	1/2	1
MCP2	1	0/1	0	0
MCP3	1/2	0	Not Determined	0
MCP3	0/1	0/1	0/1	1
MIP1b	0	0/1focal	1	0
MIP1b	1/2focal	0/1	0	0

MIP3a	1	1/2	0/1	0/1
MIP3a	2	1/2focal	1/2	0/1
MIP3 β	2	1	0	1 / 2
MIP3 β	1/2	1/2focal	2	1/2focal
RANTES	0	2	0	1/2focal
RANTES	1/2	0/1	1/2focal	0/1
mMIG	0/1	1	0/1	0/1
mMIG	0	0/2focal	0	0/1
mMDC	1/2	1/2	0	1
mMDC	0/1	2/3focal	2	2/3focal
mC10	3/4	1/2	1/2focal	1
mC10	3/4	1/2	1/2	1/2focal
vMIP1	2	0/1	0/1	1
vMIP1	2	1/2	2/3	2/3

Key: “*” used as positive controls. Each horizontal row represents a separate Rhesus macaque and the histology scores of the 4 samples assayed from each animal. “Focal” one vessel in the section showed prominent perivascular inflammation (*i.e.*, one section of the slide around a vessel showed prominent infiltration, where as the rest of the slide was more or less normal). “Center” and “Edge” refer to where the tissue section came from. “Center” tissue sections were taken at the site of the intradermal injection; “Edge” tissue sections were taken further from the injection site, towards the edge of the wheal generated by injection.

Example 4Chemokine Injection into Rhesus monkeys, Experiment 2

In a second experiment, different amounts (approximately 8, 2.4, or 0.8 μg in 100 μl PBS) of different chemokines were injected intradermally in Rhesus macaques under anesthesia. Twenty-four or 48 hours later, 6 mm skin punch biopsies were taken using aseptic technique, then bisected and prepared for analysis. One portion of the biopsy was embedded in OCT compound, flash frozen and stored at -70°C . The other portion of the biopsy was fixed in formalin and embedded in paraffin wax; subsequently, sections were stained with hematoxylin and eosin and microscopically examined for cell infiltration into the dermis (Table 6). As a negative control, monkeys were injected with PBS lacking chemokines.

Mononuclear cell infiltration in the dermis was scored as in Example 5. As indicated in Table 6, chemokines mC10 and vMCK-2 caused substantial mononuclear cell infiltration, especially at 24 hours. It was notable that a chemokine encoded by a mouse virus (vMCK-2) exhibited potent activity in primate cells. Infiltrating cells were observed predominantly in adipose tissue, although cells were also noted in the subcutaneous region and, in the case of vMCK-2 at 48 hours, in the collagen matrix of the superficial dermis. Chemokines mMDC and vMIP-1 caused less inflammation than mC10 and vMCK-2.

Table 6 Mononuclear Cell Infiltration

Chemokine		24 hr	48 hr
mC10:	0.8 µg	2	0
	2.4 µg	1	2
	8 µg	3	2
mMDC:	0.8 µg	2	0
	2.4 µg	0	0
	8 µg	2	0
vMIP-1:	0.8 µg	0	0
	2.4 µg	1	0
	8 µg	0	0
vMCK-2:	0.8 µg	2	2
	2.4 µg	2	2
	8 µg	4	3

Example 5Chemokine Injection in Rhesus Macaques, Experiment 3

In a third experiment, greater amounts of vMCK-2, mC10 and vMIP-1 (60, 20, or 8 µg) were injected intradermally in Rhesus macaques under anesthesia. As before, biopsies were taken 24 and 48 hours later, then prepared and analyzed histologically (Table 7). Samples were scored as in Example 3. As shown before in Example 4, vMCK-2 was observed to cause a dramatic infiltration of mononuclear cells as well as polynuclear cells; the 20 µg injection caused more infiltration than did the 60 µg and 8 µg injections. vMIP-1 caused a mild infiltration at all doses tested. In contrast to the previous two experiments (Examples 4 and 5) where lower chemokine concentrations were used, mC10 caused little to no infiltration in this experiment.

Table 7 Mononuclear Cell Infiltration

Chemokine	Dose	24 hours		48 hours	
		monkey 1	monkey 2	monkey 3	monkey 4
vMIP-1	60 µg	1		1	
	20 µg		1		0
	8 µg	0		0	
C10	60 µg	0		0	
	20 µg		0		0
	8 µg		1		0
vMCK-2	60 µg	3		3	
	20 µg		4		2
	8 µg		3		1
saline		0	1	0	0

To better define the identity of the infiltrating cells, samples from the last two monkey studies (*i.e.* Tables 6 and 7) were analyzed by immunohistochemistry using antibodies specific for different cell types. These antibodies included CD68 (expressed on macrophages, neutrophils and dendritic cells), MHC II (antigen-presenting cells, *e.g.* macrophages and dendritic cells), HAM-56 (macrophages), fascin (dendritic cells, endothelial cells and epithelial cells), elastase (neutrophils), cytokeratin (epithelial cells), CD3 (T cells), CD20 (B cells), and CD1a (Langerhans cells). The vMCK-2-injected skin samples contained primarily neutrophils and antigen-presenting cells, including macrophages and dendritic cells. The mC10-injected skin samples contained primarily antigen-presenting cells, including macrophages and dendritic cells, but few neutrophils. The vMIP-1-injected skin samples contained primarily neutrophils and macrophages, with few dendritic cells. Few T cells, and no B cells, were found in the skin samples for each of the three chemokines.

Example 6Chemokine adjuvant activity in Rhesus monkeys

Since the chemokines mC10 and vMCK-2 recruited antigen-presenting cells, including dendritic cells, to the site of injection, these two chemokines were tested for their ability to act as an immunization adjuvant to augmenting the immune response to a co-injected foreign antigen. Five groups of monkeys, 3 monkeys per group, were injected intradermally with chicken ovalbumin (OVA) as an antigen. The first group of monkeys received OVA alone while the second group contained OVA emulsified 1:1 with incomplete Freund's adjuvant (IFA), a standard adjuvant that is typically required in such studies to enable development of an antigen-specific immune response. The third group contained OVA plus IFA plus vMCK-2, while the fourth group contained OVA plus IFA plus mC10. The fifth group contained OVA plus vMCK-2, without IFA. Injections were done intradermally in a 100 μ l volume containing 2 mg OVA and 16 μ g chemokine. Ten ml of peripheral blood was drawn from each monkey twice a week for three weeks and the blood samples were then subjected to centrifugation over Ficoll to remove the erythrocytes and granulocytes. The plasma supernatant was analyzed by sandwich ELISA to determine the levels of anti-OVA antibodies using OVA-coated plastic dishes and a biotinylated anti-monkey IgG detection antibody (Table 8).

Table 8 Induction of anti-OVA Antibodies

	day 0	day 5	day 9	day 12	day 16	day 19
OVA	266	240	222	396	376	597
	817	549	880	757	907	1262
	87	62	106	69	87	85
OVA+IFA	35	28	103	5515	10877	10621
	71	78	58	6436	20324	25011
	93	58	89	4175	12340	20596
OVA+IFA+vMCK2	133	92	129	1194	1738	2737
	114	88	157	5581	52320	16276
	181	140	104	9215	10437	46055
OVA+IFA+mC10	95	89	125	19537	51204	32014
	107	86	129	29317	38857	32282
	144	133	129	12057	31480	34211
OVA+vMCK2	25	44	38	140	413	462
	77	89	76	330	313	275
	89	30	28	128	279	368

Key: Numbers represent the levels of OVA-specific IgG, expressed in Units/ml, in the plasma of the 15 monkeys. Each horizontal line shows the response of an individual monkey over time after immunization. To determine the levels of OVA-specific IgG, dilutions of each plasma sample were analyzed by sandwich ELISA using OVA-coated plates together with a biotinylated anti-monkey IgG detection antibody. Optical densities were converted into arbitrary Units, where a Unit is defined as the inverse of the plasma dilution that produces 50% of the maximum response from a standard curve obtained by serial dilution of an ascites collected from OVA injected mice and containing OVA-specific antibodies.

As indicated in Table 8, the monkeys injected with OVA + IFA developed a significant antibody response to OVA, as demonstrated by development of circulating anti-OVA IgG, commencing day 12. In comparison, the levels of OVA-specific IgG in the monkeys injected with OVA, mC10 and IFA were substantially greater than those in monkeys not receiving mC10. Specifically, the inclusion of mC10 lead to

approximately four-fold more OVA-specific IgG on day 12, and three-fold more OVA-specific IgG on both days 16 and 19 compared to monkeys injected with just OVA + IFA alone.

While less potent than mC10, vMCK-2 also exhibited the capacity to augment the OVA-specific antibody response. Two of the three monkeys injected with OVA + IFA + vMCK-2 exhibited significantly more circulating OVA-specific IgG than those of the monkeys injected with OVA + IFA alone, as observed on day 16 for one monkey and day 19 for the other. The third monkey failed to develop an OVA-specific IgG response that was significantly different from monkeys receiving OVA alone, most likely due to a technical error in immunization of this monkey. Monkeys receiving OVA plus vMCK-2 alone, *i.e.*, in the absence of IFA, failed to elicit a significant antigen-specific OVA response.

Example 7 (Prophetic)

This example describes a procedure to evaluate the efficacy of APC chemotaxins in augmenting systemic and/or mucosal immune responses to infectious diseases.

Groups of mice are injected either subcutaneously, intradermally, intranasally, or by any other mode with varying doses of the virus, bacterium or parasite under study, using a typical immunization schedule, *e.g.*, days 0, 7, and 14, in the presence or absence of APC chemotaxin given simultaneously with the microorganism in an appropriate formulation. Serum and/or mucosal secretions are collected on days -7, 0, 7, 14, 21, 28 and 35 for antigen-specific antibody analysis by ELISA. Mice are sacrificed at different time intervals after the last immunization to quantitate the antigen-specific antibody-forming cells and antigen-specific T cell responses (both cytotoxic and helper T cell populations) present in immune compartments, using standard procedures.

Example 8 (Prophetic)

This example describes a procedure to evaluate the efficacy of APC chemotaxins to augment anti-tumor immunity in cancer immunotherapy regimens.

While many tumor cells express unique tumor-associated antigens, these
 5 antigens are invariably weak immunogens and fail to generate potent anti-tumor immunity during tumor progression. The ability of APC chemotaxins to augment protective anti-tumor immunity can be evaluated using a model system of cancer immunotherapy in mice. In this model, mice are transplanted with a syngeneic thymoma (EL4 cells) that have previously been transfected with the experimental
 10 protein antigen OVA. Without further intervention, the tumor grows and eventually kills the mouse. Animals can be at least partially protected by vaccinating them with OVA plus adjuvant to induce an antigen-specific immune response directed against the OVA-transfected thymoma cells. This model is an effective way to evaluate the relative efficacy of adjuvants in augmenting protective anti-tumor immunity. Positive controls in this model include the following adjuvants: CFA, IFA, alum and GM-CSF. The ability of APC chemotaxins to augment cancer immunotherapy regimens can be evaluated by comparison to these known adjuvants.

Example 9 (Prophetic)Design of Hybrikines

“Hybrikines” are chimeric chemokine polypeptides designed to generate novel molecules with the appropriate, desired, and enhanced qualities. Usually, the amino acid sequence of a desired hybrikines is determined, and the molecule is produced by chemical synthesis.

25 The sequences of chemokines hMCP-2, mC10 and mMDC are conceptually divided into functional domains based on the spacing of the invariant cysteine residues. Chimeric sequences are prepared by swapping domains between these molecules. The amino termini regions (all amino acids of the mature native protein amino terminal of the first conserved cysteine residue) are swapped onto the remaining
 30 portion of a different chemokine molecule to create hybrids. As shown in Figure 2, the amino terminal regions between mC10 and hMCP-2 create two novel molecules: the mC10/hMCP2 and hMCP2/mC10 “hybridkine” molecules. In addition, the amino

terminal regions between mMDC and hMCP-2 are swapped to create an additional two novel molecules: the mMDC/hMCP2 and hMCP2/mMDC “hybrikine” molecules. These polypeptides are synthesized chemically using classical Fmoc peptide chemistry. The chemotactic abilities of these polypeptides are then tested in *in vitro* and *in vivo* chemotaxis assays.

The present invention provides novel methods and materials relating to APC-chemotactic compositions and therapeutic and prophylactic immunization. While specific examples have been provided, the above description is illustrative and not restrictive. Many variations of the invention will become apparent to those of skill in the art upon review of this specification. The scope of the invention should, therefore, be determined not with reference to the above description, but instead should be determined with reference to the appended claims along with their full scope of equivalents.

All publications and patent documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication or patent document were so individually denoted.